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Autoxidation of the Fatty Acids

3. THE OILY PRODUCTS FROM ELAIDIC AND OLEIC ACIDS. THE FORMATION OF MONOACYL DERIVATIVES OF DIHYDROXYSTEARIC ACID AND OF $\alpha\beta$-UNSATURATED KETO ACIDS

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(*Received* 8 *February* 1949)

In Part 1 (Ellis, 1932) it was shown that finely dispersed elaidic and oleic acids, when exposed to oxygen at temperatures of about 65°, showed an uptake of over 20% by weight in a few hours. About one-fifth of this oxygen was evolved as carbon dioxide and an appreciable but uncertain amount of water vapour was also given off. In Part 2 (Ellis, 1936) it was shown that the main crystalline product of these autoxidations for both acids was elaidic acid epoxide. Suberic, azelaic, heptane-1-carboxylic and octane-1-carboxylic acids were also isolated, but of the 120% yield of products of a typical autoxidation there still remained some 68–76% of oily products unidentified. The components of these oils have now been largely identified. They contain the monoacyl derivatives of dihydroxystearic acid, $\alpha\beta$-unsaturated keto acids, a colourless oil regarded as a dimeride of the latter acids, and coloured polymerized products of the unsaturated keto acids, together with small yields of peroxide and, apparently, of β-ketone-containing materials. (This term is used to denote acids in which the olefinic linkage is $\alpha\beta$ to the keto group—thus, C:C.C:O—not $\alpha\beta$ to the carboxyl group.) Further observations have been made on the amounts of carbon dioxide evolved at different stages of the autoxidation process and on the oxygen uptake in the more recent and lower-temperature experiments. Also, in the Experimental part, some account is given of improved methods whereby the autoxidations are brought about at appreciably lower temperatures than previously, resulting in very considerable changes in the composition of the products formed.

Formation of monoacyl derivatives of θι-dihydroxystearic acid (isomer of m.p. 132°). These probably occur in the oils as pairs of structural isomers and are of the type shown in the formulae:

$$\mathrm{R.CH.CHR' + R''.COOH} \quad (\text{with O bridging CH and CHR'})$$

$$\rightarrow \mathrm{R.CH(OH).CHR'.OOCR'' + R''.COO.CHR.CHR'(OH).}$$

Evidence given below shows the readiness with which elaidic acid epoxide combines additively with acids present, also in the free state, as autoxidation products. This is especially so at the higher temperatures and during the longer periods employed in the non-catalytic autoxidations. In the more recent experiments which have been conducted at temperatures of about 50° these monacyl derivatives have almost disappeared from the oily products.

Formation of $\alpha\beta$-unsaturated keto acids. The sharp, but not unpleasant, odour which invariably accompanied the saponification of the oily products and which had hitherto been attributed to traces of nonaldehyde due to oxidative fission, has now been shown to be due to a mixture of methyl heptyl ketone and nonaldehyde which could be isolated quantitatively. The source of the methyl heptyl ketone was at first thought to be a β-ketonic acid. Enol reactions were given very distinctly by some of the oily fractions but not by the light petroleum-soluble fractions, which gave the higher yields of the ketone. The presence of a 1:3-diketone was considered. This at first seemed the more probable since the pyrazoline colour reaction for such compounds was very marked. It was ascertained, however, that this reaction could be obtained by omitting the reduction process as applied to the test. It became apparent that an unsaturated ketone grouping would explain both this colour reaction and the hydrolysis not only to the methyl heptyl ketone, but also to the nonaldehyde. There evidently existed in the oily products of autoxidation, presumably in the *cis*-form from oleic acid and in the *trans*-form from elaidic acid, the two $\alpha\beta$-unsaturated keto acids:

$$\mathrm{CH_3(CH_2)_6CO.CH{:}CH.(CH_2)_7.COOH,} \quad \text{(I)}$$

and $$\mathrm{CH_3(CH_2)_7CH{:}CH.CO.(CH_2)_6.COOH.} \quad \text{(II)}$$

These compounds may be regarded as Schmidt and Claisen condensation products. (I) would be formed by the action of sodium hydroxide on azelaic semialdehyde, and methyl heptyl ketone and (II) would be formed from nonaldehyde and octan-8-one-1-carboxylic acid. The reversed reactions have been conveniently effected in this work by hydrolysis with 0·05N-sodium hydroxide, as compared with the 10% solutions used in condensations described by Schmidt (1880). Two of the four hydro-

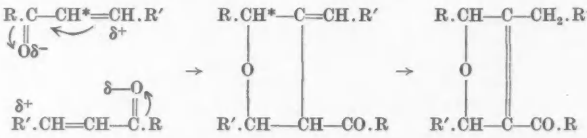
lysis products of (I) and (II) have been mentioned above. Evidence for the presence of the other two has been obtained, the aldehyde rather as a polymerized product.

The formation of these hydrolysis products fixes the positions of the keto groups. There is no evidence for supposing any movement of the double bond from the original position. Many unsaturated ketones do show $\alpha\beta \rightleftharpoons \beta\gamma$ tautomerism, but generally owing to asymmetrical grouping about the double bond, as has been demonstrated in the extensive researches on these compounds by Kon and his coworkers (see Birch, Kon & Norris, 1923). Straight-chain unsaturated ketones have been studied by Blaise (1905) and later by Eccott & Linstead (1930), who showed that the butylideneacetones exist as independent, static isomers. The catalytic action of acids and alkalis interconverts the isomers, and accordingly the same mixture of products is formed from either isomer (Farrow & Kon, 1926).

As a result of these observations it was decided to attempt the preparations of the unsaturated ketones that might be obtained by oxidation of the *cis*- and *trans*-forms of ricinoleic acid. These preparations have been effected (Ellis, 1950) and a quantitative study of the hydrolysis product of the unsaturated ketones obtained (in this case methyl hexyl ketone) has served as an indication of the concentrations of the autoxidation products. These unsaturated keto acids are contained, for the most part, in the light petroleum-soluble fraction of the oily products. This fraction is shown in the accompanying separation chart (Fig. 2) as the 'soluble oil' at the conclusion of operation 3. The oils insoluble in this solvent contain a smaller proportion of substances containing the unsaturated ketone grouping, but they also appear to contain β-ketone groups, as a result of further autoxidation. The yields of the unsaturated keto acids are set out in cols. 10 and 11, Table 1. These figures have been arrived at from the yields of methyl heptyl ketone and nonaldehyde given in col. 9 and obtained on hydrolysis of the 'soluble oil' as explained in the Experimental part.

The supposed isomeric dimerides of the η- and κ-keto-elaidic (or oleic) acids. It does not appear possible to avoid the formation of appreciable proportions of these substances in the autoxidation processes, whatever temperature is employed. They form one of a number of constituents in the 'soluble oil' referred to in the previous paragraph, providing the autoxidation has been conducted above 60°, but at temperatures between 45 and 50° this soluble oil not only represents over 90% of the products but may contain little else besides the unsaturated keto acids and these supposed dimerides. The latter are acids and contain the unsaturated ketone grouping. In this communication they are not included in the category of unsaturated keto acids which, for convenience, includes only the significant η- and κ-keto-elaidic (or oleic) acid products. They are more stable than the latter, resisting the alkaline hydrolysis which has led to the identification of these comparatively simple keto acids. This hydrolysis yields volatile and water-soluble products which can be removed and a *n*-hexane extract of the residues gives the supposed dimerides in their most concentrated form. This concentration is designated the 'colourless oil' obtained after operation 6 as shown in the separation chart (Fig. 2).

It may be thought that the experimental work so far carried out on this oily mixture of supposed dimerides (see below) does not justify the assignation of a formula at this stage. However, all observations so far made support the hypothesis that there is formed a substituted dihydrofuran ring, which may arise as a result of reactions indicated by the equations:



The first reaction is regarded as initiated by polarized effects of the electromeric displacements in two molecules. The oxygen linkage is regarded as taking place simultaneously with a migration of the hydrogen (*) to the carbonyl carbon. The second reaction is the isomeric conversion of the $\beta\gamma$- to the $\alpha\beta$-form of the unsaturated-ketone grouping. Such a conversion would be liable to take place because of the more stable form resulting by entrance of the double bond into the ring (cf. Hugh & Kon, 1927).

Polymerized oils and their β-keto-acid content. The oily products insoluble in light petroleum are shown in Fig. 2 as resulting after the conclusion of operation 3. This fraction is almost absent in the products from the lower-temperature (45–50°) processes, but autoxidation at temperatures approaching 100° will give over 70% of coloured, resinous substances. Boiling light petroleum will remove from these substances some nearly colourless oils which give definite enol reactions and which yield carbon dioxide on hydrolysis. These are regarded as β-keto-acid peroxidation products. This fraction of the oily products also contains most of the monoacyl esters from the epoxide referred to above. It therefore yields on saponification the nearly insoluble di-

hydroxystearic acid with water-soluble and volatile acids, which are the hydrolytic products of these esters and which can be separated from the remaining oily or resinous material. The higher-temperature processes yield some 50 % of these resins (Table 1, in Part 2, Ellis, 1936). They have been fractionated into substances of increasing hardness and decreasing solubility. They disappear from the products of autoxidation as the temperature employed falls, and as the yields of the unsaturated keto acids increase. It is well known that unsaturated ketones readily polymerize, and it is shown for those obtained from ricinoleic acid (Ellis, 1950) that this polymerization takes place rapidly at the higher temperatures employed in these autoxidations.

The presence of peroxides. No oily fraction of these autoxidation products has failed to give a small peroxide value as indicated by liberation of iodine from potassium iodide and acetic acid. In all cases the simple method of peroxide estimation proposed by Lea (1938) has been adopted. The results were calculated as a peroxide of oleic acid (giving mol. wt. 314) with one available oxygen atom. The average concentration found in oily products of sixteen different experiments was 1·7 % (range 0·3–3·4 %). No significance can be placed on the variations obtained because of the number of disturbing factors both in the autoxidation process itself, and in the isolation of the products examined. Some of the higher results were obtained from oils extracted from the large bulk of the dispersing medium with a considerable volume of ether. The use of this peroxide-forming solvent has been replaced, in recent experiments, by *n*-hexane, in which the whole products of the lower-temperature autoxidations, including the peroxide-containing substances, are soluble. Eleven more recent experiments have given lower peroxide figures (average 1·2 %). Nearly all these results were from catalysed experiments, but a recent preparation of carefully purified oleic acid after an oxygen uptake of 21 % by weight gave total products with a peroxide content of 1·4 %. It has been observed on a number of occasions that, on removing the source of heat from an autoxidation process, there continues a slow absorption of oxygen, and it has been ascertained that this uptake is accompanied by an increase in the peroxide content of the products, particularly after one of the lower-temperature autoxidations. It was observed that the total products from elaidic acid after an oxygen uptake of 15 % by weight at 45–55°, gave 0·9 % peroxide, but these products, on further exposure to oxygen at room temperatures for 3 days, gave a peroxide value of 1·7 %. For an experiment at 65–70° with an oxygen uptake of 20 % the products gave 1·0 % peroxide and on the 3-day exposure to oxygen at room temperatures only a small increase to 1·2 % was obtained. It is only in the first of these two experiments that an appreciable unsaturated ketone formation would be observed. The light petroleum-soluble oil, obtained after saponification of the products of autoxidation and referred to above as supposed dimerides, contains the unsaturated ketone grouping, and the peroxide-free oil will acquire a value of over 2 % on exposure to oxygen during some days at room temperatures. There is little doubt that the peroxide-forming properties of these substances must be attributed to their containing this unsaturated-ketone grouping. As mentioned above, pure unsaturated keto acids have been prepared and they give much higher peroxide values than have been obtained in any of these autoxidation products.

On the whole, the peroxide contents of the autoxidation products must be regarded as of a low value with little variation. If these peroxides are formed by an unstable addition of molecular oxygen to the unsaturated ketone grouping, in a manner such as is suggested below, they may donate *both* oxygen atoms to the substrate. Hence the average value of 1·2 % given above must be halved. It seems probable that a rise of temperature may increase their formation, but apparently owing to a mass action effect and their increased reactivity at the higher temperatures, their concentration may not materially change.

Carbon dioxide evolved during autoxidation. The more recent methods used in the autoxidation processes have lent themselves to simple and accurate means of determining the volume ratio of carbon dioxide formed to oxygen uptake. The results are given in col. 8, Table 1, and for the nine experiments in which the autoxidation of elaidic acid was carried out at temperatures below 60° the average ratio for CO_2/O_2 is 0·20, while the results recorded in cols. 5 and 6, Table 1, Part 2 (Ellis, 1936) for nine experiments, in which the temperatures were 60° and over, gave an average of 0·17. This difference is not a great one. The extreme interest in these observations is that the amount of carbon dioxide formed in the autoxidation process is not less, but actually more, at the lower temperatures employed. Elaidin (Exp. 21, Table 1) has given a higher ratio, and oleic acid of doubtful purity (Exp. 4) has given a low ratio, otherwise there is a fairly constant ratio of about 0·2 representing 20 % of the oxygen uptake being evolved as carbon dioxide. In the earlier work it was shown that appreciable quantities of water were also formed and assuming these two products to represent chain degradation ($.CH_2. \rightarrow CO_2 + H_2O$) the oxygen so evolved in the form of water and carbon dioxide must be about 30 % of the uptake.

When the autoxidation is prolonged for some period after the maximum velocity has been attained, this ratio increases from about 0·2 to 0·4. If water be included, as indicated above, the ratio must reach 0·6 and must represent 60 % of the oxygen uptake being evolved as carbon dioxide and water (Table 2).

Table 1. *Autoxidation of elaidic and oleic acids. Oxygen uptake and yields of some resulting products*

(Bracketed figures are approximate.)

No. of exp.* and method	Wt. of acid taken† (g.)	Cobalt wt. as metal‡ (g.)	Temp. (°)	Duration of exp.	O_2 uptake (% by§ (wt.)	O_2 evolved as CO_2 (% by wt.)	R.Q.‖	Methyl ketone¶ etc. (%)	η-Keto-elaidic acid (%)	η- and κ-keto-elaidic acids (%)	Remarks on different fractions obtained from total oily products or from petrol** soluble fraction of same	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	
1*a*	20·9	0·10	78	8 hr.	20·8	(2·11)	(0·10)	2·3	4·2	8·3	Fraction soluble in petrol**	
2*a*	5·22	0·03	100	8 hr.	25·5	6·21	0·24	Nil	Nil	Nil	Different fractions examined	
3*b*	4·6	0·07	50	4 days	7·5	—	—	3·3	6·0	11·9	From oleic acid	
4*b*	4·4	0·02	37	6 days	11·8	1·86	0·16	3·1	5·6	11·2	Similar to 3. See text	Total oily products
5*b*	3·0	Nil	63	3 days	17·8	—	—	2·1	3·8	7·6	Unchanged acid present	
6*c*	4·45	0·02	53	7 hr.	12·8	2·67	0·20	3·1	5·6	11·2	A similar fraction	
7*b*	10·9	0·1	48	2 days	15·3	3·20	0·21	6·8	12·3	24·6	Unchanged acid removed	
8*a*	47·6	0·20	60	24 hr.	13·9	—	—	6·0	10·8	21·7	Whole product	
9*a*	105	Nil	68	2 days	13·2	2·29	0·17	6·0	10·8	21·7	Not further fractionated	Petrol** soluble fractions of oils, being nearly total oils for experiments at lower temperatures
10*a*	20·5	0·05	63	24 hr.	18·2	2·96	0·16	8·0	14·4	28·9	Fractionation of Mg salts	
11*a*	22·1	0·05	60	20 hr.	13·6	—	—	5·5	9·9	19·9	From oleic acid	
12*a*	20·2	0·02	60	24 hr.	17·8	—	—	10·0	18·0	36·0	Ether-soluble Pb salt	
13*b*	29·7	0·3	46	19 days	23·6	4·90	0·21	7·2	13·0	26·0	Unchanged acid present	
14*e*	20·0	0·02	55	5 days	23·9	4·24	0·18	12·3	22·1	44·2	Autoxidation of an emulsion	
15*b*	10·1	0·03	45	4 days	21·2	—	—	(12·0)	(21·5)	(43·0)	Water soluble Mg salts removed	
16*e*	4·2	0·02	50	2 days	13·6	—	—	8·4	15·1	30·2	Autoxidation of an emulsion	
17*b*	4·5	0·05	48	2 days	12·6	2·60	0·20	15·1	27·1	54·2	Chromatographic separation	
18*b*	3·2	0·02	48	2 days	9·2	—	—	15·1	27·1	54·2	A similar process	
19*b*	24·1	0·1	48	3 days	17·4	3·37	0·19	12·2	21·9	43·8	Solids deposited below 0°	
20*b*	47·8	0·2	50	—	17·3	3·60	0·21	25·4	45·5	91·0	Girard's reagent separation	
21*b*	20·4	0·5	50	7 days	11·7	4·77	0·41	4·3	7·8	15·5	Elaidin, partial autoxidation	
22*c*	4·56	0·02	50	3·5 hr.	6·8	1·25	0·20	11·4	20·5	41·0	Autoxidized at 2 atm. pressure	
23*c*	2·68	0·02	55	20 hr.	10·7	1·91	0·18	8·4	15·1	30·2	A similar experiment	
24*d*	3·13	0·02	(50)	6 hr.	12·3	2·68	0·22	(6·0)	(10·8)	(21·5)	Heat from an electric lamp	
25*d*	1·07	0·01	58	28 hr.	22·7	3·89	0·17	11·9	21·3	42·6	A similar experiment	
26*a*	41·8	0·2	68	14 hr.	18·4	—	—	14·1	25·3	50·6	Volatile acids removed	

* Letters *a*, *b*, *c*, *d* and *e* refer to the methods of autoxidation (p. 133).
† Weights in g. of elaidic acid unless oleic acid is referred to.
‡ Present as cobaltous elaidate or oleate.
§ Percentages refer to weights in col. 2 irrespective of the presence of any unchanged acid in the products.
‖ Ratio of carbon dioxide formed to oxygen uptake, or CO_2/O_2.
¶ Methyl heptyl ketone, nonaldehyde and its acid. Products of hydrolysis of fractions noted under 'Remarks' column.
** 'Petrol' signifies the use of light petroleum (b.p. 40–60°) or of *n*-hexane. See separation chart, Fig. 2.

Oxygen uptake in the autoxidation process. Ample evidence was obtained in Parts 1 and 2 (Ellis, 1932, 1936) for regarding at least 20 % by weight as the oxygen required to bring about the disappearance of elaidic acid in this process. In col. 6, Table 1, are set out further records of oxygen uptake. In most of these experiments the autoxidation has been stopped to avoid possible destruction of the initial products sought. In those cases unchanged elaidic acid was observed and often separated by the processes shown in Fig. 2. When an uptake of a little over 20 % had been obtained the autoxidation was always stopped, because it was realized that only traces of unchanged acid would be present, moreover, there was at this stage a marked falling off of the rate of uptake (cf. Ellis, 1932). In Exp. 21 the uptake was 12·6 % and the recovered acid was 50·4 % with possible traces of the epoxy acid. This result indicates an uptake of over 24 % for complete autoxidation. If the molecular uptake were 4 atoms of oxygen a 23·7 % addition by weight would be required.

EXPERIMENTAL

Apparatus employed

The large bulk of sand used in the earlier work to disperse the acids required a continuous current of O_2 to displace the CO_2 formed in the autoxidation. It was thought that this displacement was uneven owing to pockets of resistance to the current being set up in the sand. An unsteady O_2 uptake would thereby result. It was therefore decided to devise a method which eliminated the use of sand. However, some experiments, marked (*a*) in col. 1, Table 1, were made with the earlier method.

Autoxidation is a surface reaction taking place very slowly when O_2 is passed into solutions of elaidic or oleic acids in organic solvents, with or without a catalyst, nor when O_2 is passed through aqueous colloidal solutions of the soaps of these acids. In order to obtain the initial products of autoxidation as free as possible from secondary products, fine dispersion is of paramount importance. A fine emulsion of the acid should, therefore, be most suitable. Such experiments with fine emulsions have been used and are marked (*e*) in col. 1, Table 1, but the difficulty of handling sufficiently large quantities and of separating the products from the emulsifier has not warranted extensive use of this method.

Most of the experimental results recorded in Table 1 have been obtained by use of a simple form of apparatus consisting of the common type of desiccator of about 16 cm. diam. In the bottom half is placed NaOH to absorb the CO_2 formed. The lid is provided with clamps to make it gas-tight. Its central opening is fitted with a rubber bung through which passes a thermometer and two tubes, one of which reaches nearly to the bottom of the desiccator. The tubes are for displacing air and for supplying O_2. Thin sheets of filter paper are reduced to a third of their length by folds of about 5 mm. depth and are cut into strips of a width equal to the depth of the upper part of the desiccator. These cuts are made just off right-angles with the folds so that by reversing alternate layers of the strips they are prevented from falling into each other's folds. A drum is thereby formed of considerable surface area and of a size to fit loosely into the desiccator over a wide-meshed gauze. A dispersion of about 1 mg. of the acid to be autoxidized to 1 cm. surface was generally allowed for. In dispersing elaidic acid over the paper the catalyst (cobalt elaidate) is at first dissolved to a clear solution in the melted acid and this solution is diluted with sufficient light petroleum to cause dispersion over the entire paper of the drum. This dispersion is carried out in a suitable beaker, and immediately the solution has been absorbed the drum must be taken out and the paper strips spread out for evaporation of the solvent. Evaporation from the beaker draws most of the acid to the upper edges of the paper strips. The drum is finally re-assembled and placed in position in the desiccator, which is supplied with O_2 from a graduated gas holder and brought to the required temperature (48–55°) in an oven or incubator. Experiments with this apparatus are marked (*b*) in col. 1, Table 1.

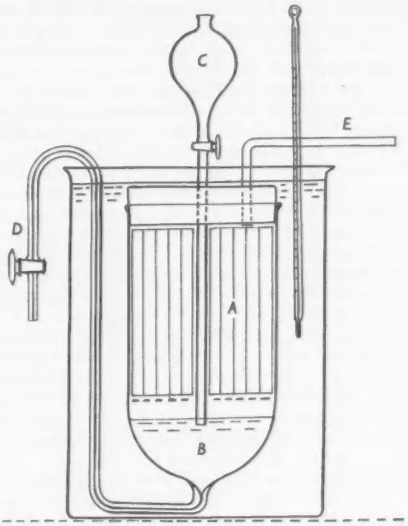


Fig. 1. Apparatus for autoxidation. *A*, folded filter paper; *B*, NaOH solution; *C*, dropping funnel containing NaOH; *D*, tap; *E*, oxygen-supply tube.

A modified form of this apparatus (Fig. 1) has been used in the smaller-scale experiments. It was designed especially to enable periodic determinations of the CO_2 formed during a prolonged autoxidation as described below. The desiccator is replaced by a glass cylinder drawn in and fused with a capillary tube for ready removal of the alkali without dismantling the apparatus. It is heated in a beaker of water and contains the drum of fluted paper strips supported by a glass grid over the alkali.

A third, slightly modified, form (*c*) of the last described makes use of a wide-mouthed bottle. The stopper is of metal, well clamped to the neck so as to carry out an autoxidation at increased pressures. The results obtained are recorded in Exps. 22–23 of the table. Yet another slight modification (*d*)

was made to enable an autoxidation to be carried out in the presence of artificial light, which also provided the source of heat. Little work has been done with this form. The wide bottle contains in its centre a low-powered electric lamp and the drum of fluted papers is replaced by a single, deeply fluted paper lining the inner side of the bottle. The most convenient form of this modification has been to use a bright tin canister of 12·5 cm. diameter. A 15 W. lamp gives the required temperature near the heavy flutings of the paper. An 'opal' lamp seems to give a more even distribution of heat than does one of clear glass. The alkali is placed in a Petri dish, the sides of which support a metal ring of 1 cm. width which, in its turn, supports the paper lining fluted to similar dimensions. The results of Exps. 24 and 25 given in Table 1 were obtained by use of this form of apparatus.

Monoacyl derivatives from elaidic acid epoxide. Experiments relating to these esters were conducted to ascertain the proportion they formed of the oily products. The unfractionated oils obtained by autoxidations carried out at over 60° yielded about 12% of *threo*-dihydroxystearic acid, m.p. 133°. Some oily fractions after saponification with aqueous NaOH and acidification gave precipitates containing little solid matter. This was nearly always the case when oils obtained at the lower temperatures (45–55°) were saponified. Other fractions of oils obtained at over 60° and so treated gave precipitates that contained so much of the solid dihydroxystearic acid as to be readily filterable through a small paper disk. After washing such a precipitate with water, drying and removing any oil with a minimum volume of ether, the dihydroxystearic acid was nearly pure and could be weighed for its estimations. One crystallization from methanol yielded the product with its correct m.p. None of its isomer of m.p. 95° has been identified.

The esters giving rise to dihydroxystearic acid on hydrolysis are therefore only formed at the higher-temperature autoxidations, under which condition the free elaidic acid epoxide is often present in the products to the extent of over 16% (Table 1, Part 2, Ellis, 1936), but in the lower-temperature autoxidations its concentration is much less, and in Exp. 19 (Table 1), carried out at about 48° for 3 days, only 4·1% of the epoxide was isolated.

At the higher temperatures, and for the duration of autoxidations so conducted, it has been possible to demonstrate that ester formation readily occurs between the epoxide and certain of the free acids found in the products of those autoxidations. Molar proportions of the epoxide and octane-1-carboxylic acid were heated at 65–68° for 5 days and gave about 60% of an oily product. It was purified by crystallizing out the unchanged epoxide from cold light petroleum and removing the unchanged acid by steam distillation. The product was soluble in light petroleum and gave an equiv. wt. of 509 (theory 465 for a mixture of the two isomeric esters). Saponification yielded 66% of dihydroxystearic acid, m.p. 133° (theory requires 69%). The epoxide esterifies by intermolecular reaction when heated alone under these conditions. This accounts for the high equiv. wt. A specimen of equiv. wt. 299 heated to 68° for 12 hr. gave an equiv. wt. 321. Heated at 68° for 16 days it gave a thick oil of equiv. wt. 564. This substance gave evidence of some polymerization. Similarly, the epoxide was found to combine readily with azelaic acid, another of the scission products of autoxidation. Thick oils, insoluble in light petroleum, were obtained but they were not further investigated. Deatherage & Mattill (1939) have shown that monoacetyl derivatives are formed by heating autoxidation products of different monenes with acetic acid.

Oils from one of the higher-temperature autoxidations were dissolved in ethanol and water was added until most of these oils were thrown out. They were thereby freed from the dibasic scission products azelaic and suberic acids, for these acids are very soluble in dilute ethanol. This process was followed by washing with warm water. On saponification they yielded a further amount of both acids which were separated and identified by the methods described in Part 2 (Ellis, 1936) for the same acids present in the free state. These acids are the strongest present and are the more liable to esterify with the epoxide present. The insolubility of these esters in light petroleum, as compared with the solubility of others that are formed with octane-1-carboxylic acid, accounts for the much higher yields of dihydroxystearic acid when the light petroleum-insoluble fractions of the oils were saponified than are obtained from the soluble fractions. In Exp. 12, Table 1, Part 2 (Ellis, 1936) an autoxidation carried out at 68° was shown to give 15·1% of the free elaidic acid epoxide. Subsequently, it was found that the oils, which were given as 30·5% soluble in light petroleum and 36·7% insoluble, yielded on saponification 11·0 and 23·6%, respectively, of dihydroxystearic acid. These figures represent a formation of about 26% of elaidic acid epoxide, free and esterified.

From the above results it is evident that for the higher-temperature autoxidations not only must the epoxy acid formation be much higher than reported in Part 2 but so also that of the acid scission products. The latter were reported as totalling a 16–20% yield and suberic acid occurred in largest amount; possibly heptane-1-carboxylic acid predominated as the monobasic acid product. The total formation may be regarded as reaching about 30%.

Concentration and identification of the unsaturated keto acids. The accompanying separation chart (Fig. 2) summarizes the preliminary processes of fractionation by which the products of autoxidation were generally resolved into their components. In operation 1 the products are dissolved in 2–3 times their bulk of 96% methanol or in 3–4 times their bulk of 80% ethanol. The separation of the crystalline deposit through a Büchner funnel presents no difficulty. The second operation gives a fine emulsion, but when the mother liquors are not required the addition of a little anhydrous Na_2SO_4 assists the complete deposition of the oily matter. In the third operation the mutual solvent action of the oils becomes apparent. They are completely soluble in a small proportion of light petroleum, but precipitation of some of these oils on the addition of more solvent seems to proceed indefinitely. The solution has generally been obtained by the addition of at least 10 vol. of the solvent to 1 vol. of the oils. This solution becomes clear on standing for some time. On evaporation, using reduced pressure during the last stages so as to avoid unnecessary heat, there remains a clear light-yellow oil which readily solidifies below 0°. It may form 90% of the autoxidation products of the lower-temperature experiments of short duration, while in the higher-temperature experiments it may represent as little as 10%, being replaced by the formation of a large proportion of the coloured, resinous oils, by a large proportion of the oily monoacyl esters derived from the epoxide formed, and by an appreciable proportion of the volatile scission products already referred to. For the

majority of experiments of recent work, found the most convenient for operation, yields of 60–70% of this fraction have been obtained.

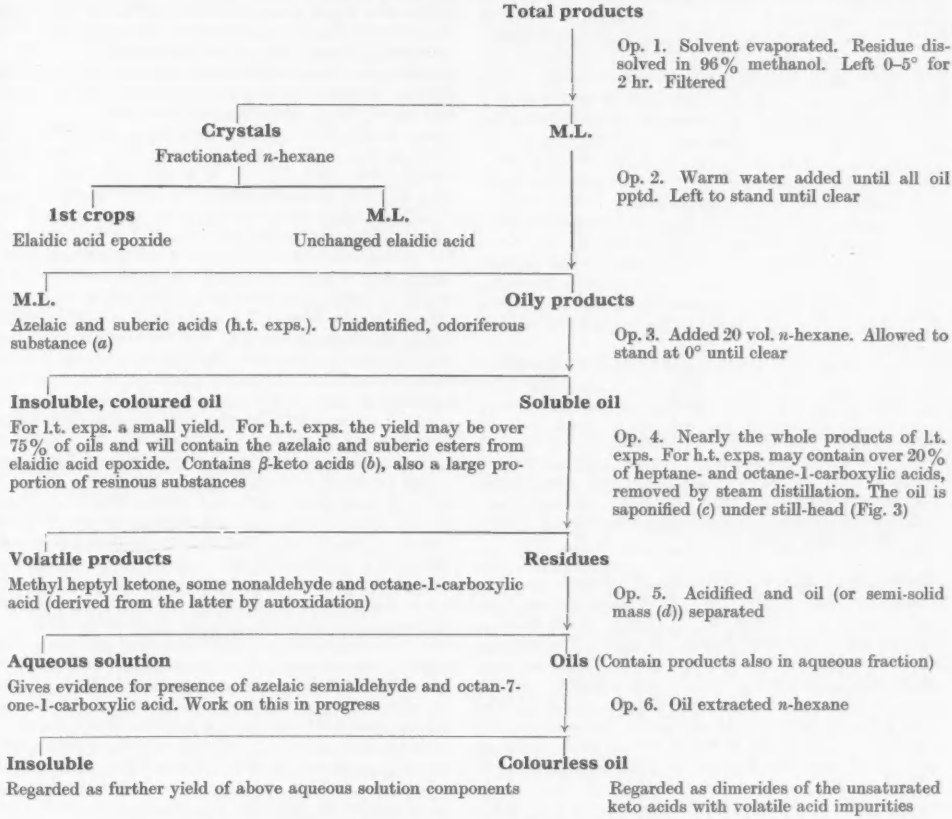
under cool conditions, in order to obtain the soluble oil, tends to leave the β-keto acids amongst the coloured insoluble oils.

Fig. 2. SEPARATION CHART

Showing preliminary separation of autoxidation products

(Abbreviations: h.t. exps. refer to those conducted above 60°; l.t. exps. refer to those conducted below 60°; Op. = operation; M.L. = mother liquor.)

Dispersing medium extracted with ether, or (for l.t. exps.) with *n*-hexane, yielding:


Total products
Op. 1. Solvent evaporated. Residue dissolved in 96% methanol. Left 0–5° for 2 hr. Filtered
Crystals
Fractionated n-hexane
M.L.
Op. 2. Warm water added until all oil pptd. Left to stand until clear
1st crops
Elaidic acid epoxide
M.L.
Unchanged elaidic acid
M.L.
Azelaic and suberic acids (h.t. exps.). Unidentified, odoriferous substance (a)
Oily products
Op. 3. Added 20 vol. n-hexane. Allowed to stand at 0° until clear
Insoluble, coloured oil
For l.t. exps. a small yield. For h.t. exps. the yield may be over 75% of oils and will contain the azelaic and suberic esters from elaidic acid epoxide. Contains β-keto acids (b), also a large proportion of resinous substances
Soluble oil
Op. 4. Nearly the whole products of l.t. exps. For h.t. exps. may contain over 20% of heptane- and octane-1-carboxylic acids, removed by steam distillation. The oil is saponified (c) under still-head (Fig. 3)
Volatile products
Methyl heptyl ketone, some nonaldehyde and octane-1-carboxylic acid (derived from the latter by autoxidation)
Residues
Op. 5. Acidified and oil (or semi-solid mass (d)) separated
Aqueous solution
Gives evidence for presence of azelaic semialdehyde and octan-7-one-1-carboxylic acid. Work on this in progress
Oils (Contain products also in aqueous fraction)
Op. 6. Oil extracted n-hexane
Insoluble
Regarded as further yield of above aqueous solution components
Colourless oil
Regarded as dimerides of the unsaturated keto acids with volatile acid impurities

Notes. (*a*) Odour strongly reminiscent of that of the gorse flower.
(*b*) I.e. 2:10-diketoelaidic acid. (See text, p. 138.)
(*c*) Or treated with a view to further concentration of its unsaturated ketone content.
(*d*) In h.t. exps. esters of the epoxy acids may yield 8:9-dihydroxystearic acid.

This main product is shown in Table 1 and Fig. 2 as the 'soluble oil'. It contains nearly the whole of the unsaturated keto acids. It may contain some of these acids which have undergone β-oxidation. Such unsaturated diketo acids are less soluble in light petroleum than are the unsaturated keto acids themselves. Using a large volume of the solvent

The identification of the unsaturated keto acids and the determination of their concentration in the soluble oil have been effected by saponification of this oil and by subsequent isolation of the different hydrolysis products. An outline of the processes involved is shown in the remaining operations given in Fig. 2. By means of the apparatus described below

it has been possible to experiment on a much smaller scale than might have been necessary. The methyl heptyl ketone with some nonaldehyde and octane-1-carboxylic acid formed therefrom, is isolated during saponification by use of the still-head shown in Fig. 3. A flask (*B*) of about 400 ml. capacity is connected by a ground joint to the reflux tube (*C*) which has a length of about 30 cm. and a bore of about 11 mm. The U-tube (*E*) has a bore of 5 mm. (not less) and the arm connecting it with the reflux tube should be of 6–7 mm. bore. It will be observed that the upper portion of the fused connexion made between this arm and the reflux tube has been rounded off. This shaping is essential in

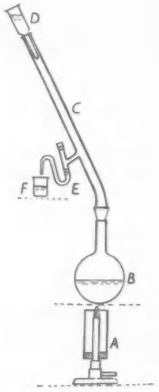


Fig. 3. Diagram of still-head for isolating small quantities of steam-distillation products. *A*, bunsen flame protected from draught; *B*, saponification flask with ground-glass fitting; *C*, air-cooled reflux condenser; *D*, condenser; *E*, inner arm containing distilled oil over water; *F*, beaker to collect water distillate.

directing the condensed methyl ketone products from the reflux tube to the U-tube. The slope of the reflux should be about 27° to the vertical. The ebullition of the reaction mixture in the flask must be slow and steady. The addition of pumice stone, or the like, is essential. So, too, is an easily adjustable flame protected from draughts. In this manner the steam line in the reflux tube can be regulated to come a few cm. above the U-tube outlet. The reflux tube is not, of course, hermetically sealed, but the small tube containing cold water serves to limit convection currents in the reflux and provides an emergency condenser in case of overheat applied to the flask. The whole condensing and collecting parts of the still-head must be thoroughly clean and free from grease before a saponification is started. If this precaution has been taken the condensed steam will run back almost entirely as a continuous film, while the condensed volatile products will run as droplets over the water film into the U-tube to form an upper layer over the water with which the tube has been filled. Any water entering the U-tube finds its way through the oil layer to the beaker (*F*). This water is used from time to time for flushing down the reflux tube. A long Pasteur pipette is used for this purpose and a shorter one is used to convey the oily layer to a weighing bottle. The U-tube must be fused to the reflux tube so as to allow of the approach of this pipette. The efficiency of this simple apparatus has rendered it almost indispensable throughout the course of these investigations on the ketonic products of autoxidation, and the technique may prove equally effective in other spheres of work. The accuracy of the method is over 90% for the small quantities examined: 0·0143 g. of methyl heptyl ketone distilled with 0·05 N-alkali (the excess used in the saponifications) yielded, in the above described apparatus, 0·0133 g. or 93%. Nonanol (0·1191 g.) gave 0·1090 g., or 91·6%. Nonaldehyde (0·1722 g.) gave from water distillation 0·1550 g., or 90·0%. Distilled from alkali, aldehydes gave low yields as will be considered below.

The other factor upon which this method of computing the concentration of an unsaturated ketone depends is the quantitative nature of the actual formation of these volatile products on alkaline hydrolysis. It has been possible to prepare heptadec-9-en-11-one-1-carboxylic, which should bear close analogy with those in these autoxidation products. It gave 32% methyl hexyl ketone (Ellis, 1950). The question also arose as to the proportion of the methyl heptyl ketone from (I) and of nonaldehyde, or its acid, from (II). The aldehyde imparted a Schiff reaction to the volatile saponification products, but it was seldom very pronounced. The slow hydrolysis by the 0·05 N-alkali used, and the consequent long exposure of small quantities of the aldehyde to air in the condenser of the still-head described, brought about nearly complete autoxidation to octanecarboxylic acid. This acid is appreciably soluble in water, but as the aqueous distillate was repeatedly used during saponification to flush down the volatile products so as to bring them into the U-tube for collection, most of the acid appeared as part of the volatile oil and was eventually removed and weighed. The octanecarboxylic acid content of these oils was determined by titration of a weighing-bottle collection from a number of experiments: 0·3649 g. required 7·7 ml. of 0·1 N-sodium hydroxide, corresponding to 33% of octanecarboxylic acid. Also 0·3729 g. required 9·15 ml., equal to 39%. A third collected lot gave 36% the average of the first two. The recovered acid gave equiv. wt. 162; calc. for octanecarboxylic acid, 158. A Zn salt gave the characteristic long plates of m.p. and mixed m.p. 131°. It was evident that nonaldehyde was one of the saponification products of the soluble oil, but the action of the alkali on it and its susceptibility to oxidation allow of only a very approximate estimate of its yield. Experiments showed that the 0·05 N-alkali used in the saponification processes brought about considerable decomposition of pure nonaldehyde when this was distilled in the apparatus described: 0·0689 g. gave 0·0350 g., a recovery of 51%; 0·0988 g. gave 0·0564 g., a recovery of 57% and from 0·0348 g. a trace only was recovered. Titration of the distillate from the last gave 11% octanecarboxylic acid and, on acidification of the flask contents and distilling, acid equivalent to a further 75% of the aldehyde was obtained. Had larger amounts of material been used in these estimations the losses of the aldehyde would have been of less significance, but it has been the practice to use about 0·25 g. as more suitable for the particular apparatus described, and in some cases less material was available.

The 36% of octanecarboxylic acid in the saponification distillates could have had no other source than that of the nonaldehyde. Octanecarboxylic acid was often in the oils examined because it was often one of the scission products of an autoxidation. In an experiment with this acid in the presence of 0·05 N excess alkali no hydrolysis of its sodium

salt on 2 hr. distillation was found since no acid appeared in the distillate. It is evident from the experiments with nonaldehyde described that the 36% of its acid in the distillates, with traces of the aldehyde itself present, must, owing to loss, represent appreciably more nonaldehyde as having been formed by hydrolysis of the η-ketoelaidic (or oleic) acid in the sample examined. It is probable that this aldehyde is actually liberated in larger amounts than is the methyl heptyl ketone from the κ-keto acid. This ketone represents the remaining 64% of the distillate. Suberic acid, which has been referred to as the most abundant of the scission products of an autoxidation (Part 2), provides further evidence for the predominance of the η-keto acid, since this dibasic acid suggests cleavage at $C_{(7)}$.

Methyl heptyl ketone is readily purified from the remaining 64% of the distillate, which sets to a crystalline mass on the addition of sodium hydrogen sulphite. Filtered, pressed and washed with a little ether, this mass has been distilled with sodium carbonate in the above-described apparatus which conveniently isolates the pure product. (Found: C, 75·9; H, 12·7. Calc. for $C_9H_{18}O$: C, 76·0; H, 12·8%.) The semicarbazone crystallized from ethanol gave well-defined plates, identical in microscopic appearance with those of an authentic specimen. It gave m.p. and mixed m.p. 119°.

The total distillation products obtained during a saponification are set out for a number of experiments in col. 9, Table 1. They are expressed as a percentage of the oil examined. The deduced concentrations of the κ-ketoelaidic (or oleic) acid are given in col. 10. These figures have been arrived at as follows. From evidence given above the total distillate reduced by 64/100 gives the percentage of methyl heptyl ketone yields. As mentioned above it was shown that heptadec-9-en-11-one-1-carboxylic acid gave 32% methyl hexyl ketone, theory requiring 43%. Hence, from close analogy, the above percentage of 64/100 may be increased to $64/100 \times 43/32$. Since the κ-keto acid (mol. wt. 296) is hydrolysed to 1 mol. of the methyl heptyl ketone (mol. wt. 142) the percentage concentration of the oil under examination becomes $64/100 \times 43/32 \times 296/142$. The figures given in col. 10 are therefore obtained by multiplying those of col. 9 by this factor. Assuming that the unsaturated η-keto acids are formed in equal amounts the total of these acids as given in col. 11 are double those of col. 10.

Further concentration of the unsaturated keto acids. No attempts have been made to concentrate the small yields of these acids in the products obtained by autoxidations carried out at over 70° (see Exps. 1 and 2, Table 1). Below 70° the 'soluble oil' (see Fig. 2) may be a complex mixture. The mutual solubility of its constituents is very high. The removal of any one set such as the volatile scission acids (heptane- and octane-carboxylic) by steam distillation or by the greater solubility of their Mg salts in water, makes possible further effective fractionation in light petroleum. Exps. 15 and 26 give results illustrating this concentration. The Ba salts, and to a less extent, the Pb salts of the unsaturated keto acids are more soluble in ether than those of the other constituents of the oil, but the manipulations involved in this separation bring about too much decomposition of the required acids (Exps. 10 and 12). The very high solubilities of the unsaturated keto acids in apparently all the common organic solvents led to their concentration in the mother liquors of cooled solutions, even when the solutions were cooled below −50°. The supposed dimerides seem, however, to possess a similar solubility.

As would be expected, the soluble oil from the lower-temperature processes, containing, for the most part, only the unsaturated keto acids and their supposed dimerides, has afforded the highest concentrations of these acids so far obtained, and it is probable that chromatographic methods will eventually effect a complete separation of these constituents. In an experiment the oil (1 g.) was dissolved in *n*-hexane and passed through a column (15 cm. × 10 mm.) of silica, on the top of which was anhydrous Na_2SO_4 (2 cm.). The filtration and elution was conducted at 0° and after some hours four bands could be distinguished. These were separated. The top one contained a trace of coloured oil, the second was an oil giving 10·5% of the volatile products (methyl heptyl ketone and nonaldehyde), the third gave an oil yielding 15% of the volatile products, answering to about 54% of the unsaturated keto acids (Exp. 17). Girard's reagent does not react smoothly with the unsaturated ketones. It reacts with nearly the whole of this soluble oil, but to give substances which are not easily hydrolysed. The most advantageous conditions of employing this reagent for separation of these ketones may not have been arrived at. The oil (4·4 g.) warmed for a few minutes with the reagent (1·7 g.) in dry ethanol (10 ml.) and acetic acid (5 ml.) yielded only 0·54 g. of an ether extract after dilution and partial, ice-cold, neutralization. This extract gave 25% of the methyl ketone products, corresponding to a 91·7% concentration of the unsaturated keto acids. Other slightly lower concentrations have been effected by these means.

The yields shown by the different fractions given in Table 1 are not closely comparable. There are too many factors involved, not only in the autoxidation process but in the isolation of the different fractions examined. The comparatively low results obtained for oleic acid (Exps. 3, 4 and 7) are due to the difficulty in removing the unchanged acid from the products.

Separation and examination of the supposed dimerides of the unsaturated keto acids. This fraction is shown in Fig. 2 as the colourless oil obtained after operation 6. It is slightly coloured when isolated from the products of the higher-temperature autoxidations. It is not easily freed from unchanged acid, from traces of heptane- and octane-1-carboxylic acids and other impurities. Since the supposed dimerides are regarded as being formed by combination of any two of the two unsaturated keto acids forming the main products of autoxidation, there may be present in the mixture six dimerides, which are not likely to lend themselves to separation. This mixture of supposed dimerides retains its unsaturated ketone groupings after being subjected to the alkaline hydrolysis which destroys η- and κ-keto elaidic or oleic acids. How far it will remain unaffected by more concentrated alkali than the 0·05N-solutions employed has not been ascertained.

Taking into consideration the impurities present, analyses agree fairly well with the proposed structure. (Found: mol. wt. 424. Calc. 593. Equiv. wts. have varied between 240 and 302 (calc. 296).) The value 302 was obtained by subjecting the oil to prolonged steam distillation which volatilized most of the impurities. Iodine values for a number of different preparations varied from 36 to 50 with an average of 45%. This points to a normal addition (calc. 43%) which is not always the case with the unsaturated ketone grouping. A carbonyl determination by the author's method (Ellis, 1927) was less affected by the impurities, as might be expected. It gave 4·8% CO (calc. 4·7%). An

approximate determination of hydroxyl by Grignard reagent in ether gave 4·3%. It points to an absence of an alcohol group since the carboxyl groups alone require 5·7% OH.

When recently prepared from a saponification process this oil has no peroxide value. Left in open beakers it soon acquires one. This O_2 uptake has been studied quantitatively. A specimen (0·1772 g.) was dispersed on filter paper and placed in a tube filled with O_2 and fitted with a calibrated Hg manometer. At the bottom of the tube was placed a saturated solution of $Ba(OH)_2$. The tube was placed in diffused light and the temperature during about 8 weeks was near 17°. The O_2 uptake was about 12 ml. (nearly 10% by wt.). The $Ba(OH)_2$ solution contained a ppt. which dissolved with effervescence on adding HCl. The filter paper was extracted with peroxide-free ether. The extract was no longer completely soluble in *n*-hexane, and contained some crystalline matter. The *n*-hexane-soluble fraction (0·1317 g.) gave a peroxide value of 2·3%, calc. as that of a peroxide of the supposed dimerides. If both oxygens were available this value must be halved. The insoluble fraction gave a value of 3·4%.

This property of peroxide formation and a consequent self-peroxidation has been demonstrated for a pure unsaturated keto acid (Ellis, 1950). The intense pyrazoline colour reaction, which is given by the unsaturated keto acid fractions is also given by these supposed dimerides. The bluish purple of the former is replaced by a reddish purple of the latter.

The presence of β-keto acids. Definite enol reactions have been obtained with some of the fractions of the oily products. They have given intense red or bluish red colours in ethanol solution with $FeCl_3$ and an immediate deep blue with the Folin-Ciocalteu reagent. These are fractions soluble in hot light petroleum. They may be obtained as a deposit from a solution of oils in this solvent, to which more of the solvent has been added.

All of the oily fractions obtained in this work have given a saponification value regarded as due to three causes: (1) The presence of the monoacyl esters derived from the epoxy acid. Their concentration may give a value nearly equal to that of the free acid. (2) A Cannizzaro reaction on the aldehydes resulting from the hydrolysis of the unsaturated keto acids and also the formation of acids by some oxidation of these aldehydes. (3) The presence of enol-reacting substances, regarded as the β-keto acid products.

For confirmation of (3) it has been necessary to demonstrate the liberation of CO_2 during a saponification process. This work has involved the carrying out of some 100 such processes and in many cases the final titration has resulted in small liberations of CO_2. Excess of the H_2SO_4 could be added and a permanent colour (phenolphthalein) restored by heating the solution. The gas could be detected in the flask after such treatment. A few quantitative experiments have been conducted: a fraction deposited from a light-petroleum solution by further addition of the solvent (1·8513 g.) was heated with excess of H_2SO_4 after its saponification. CO_2-free air was drawn through the apparatus fitted to a condenser to arrest any heptane- or octane-1-carboxylic acids. It was then passed into 0·1 N-alkali. The excess was determined (3·0 ml.). A blank experiment, with the reagents only, gave 0·3 ml. The difference, calc. as β-ketoelaidic acid, gave 4·3% in the oil. From another autoxidation a similar fraction (0·5142 g.) gave $BaCO_3$ (0·2220 g.) by passing the CO_2 through a solution of $Ba(OH)_2$. A blank experiment, with the reagents only, gave 0·0070 g. The difference calc. to the keto acid gave a concentration of 4·7%. The carbonate precipitates gave clear solutions in a few drops of HCl. Two other fractions of oils, not giving definite enol reactions, gave only 2 and 1% of the keto acid. All the concentrations are low. The fractions examined came from autoxidations of less than 20% O_2 uptake. It is reasonable to suppose that further autoxidation would give higher formations indicating this β-oxidation, just as prolonged autoxidation has been shown above to give larger yields of CO_2 itself.

Ratio of oxygen uptake to carbon dioxide evolved. The results obtained for the R.Q. of autoxidizing elaidic acid are given in column 8, Table 1. The O_2 uptake and CO_2 formed, from which the ratios CO_2/O_2 were obtained, are shown in the previous columns of the Table. The CO_2 figures have been arrived at by titration of the excess alkali in the autoxidation chamber after the O_2 uptake, or by liberating the gas from conc. alkali so placed. Both alternatives give almost identical results, since there are apparently no other volatile acids absorbed by the alkali. The CO_2/O_2 ratio has been followed during the autoxidation of elaidic acid for a period of 39 days. The apparatus used has been described above, and is shown in Fig. 1. The alkali (5–10 ml. N) was introduced by way of the dropping funnel (*C*) and washed down. After a period the O_2 uptake was read and the alkali forced out of *B* by way of the exit *D*, rinsing with water through *C*. The excess alkali was determined and the amount of CO_2 deduced. The results are shown in Table 2. The method has few sources of error, but movement of the apparatus has caused the alkali to contact the dispersion media (pleated filter papers) and give a slightly higher result for the CO_2, as is shown for the 5-day period in the Table.

These results show clearly that the R.Q. of autoxidation is comparatively low during the initial stages, as would be expected, and that beyond these stages the ratio is fairly constant at about 0·41. Since water is also given off freely, also from the early stages (Part 1, Ellis, 1932), these two chain degradation products must account for over 60% of the O_2 uptake. It will be noticed, however, that the rate per day of O_2 uptake as shown in Table 2, line 5, are those of a die-away curve similar to the actual curve given in the graph shown in Part 1. The nearly complete cessation of O_2 uptake is accompanied by the disappearance of the unsaturated keto acids. The autoxidation products contain about 17·7% of the soluble oil, which on saponification gives 3·6% volatile products with very little odour of the

Table 2. *Oxygen uptake and carbon dioxide formed by 2 g. elaidic acid during 39 days*

Successive periods (days) ...	1	1	2	2	2	3	5	7	11	5	Totals
O_2 (ml.)	75	95	71	42	44	64	58	51	72	22	594, 42%*
CO_2 (ml.)	12	20	19	16	16	27	27	21	28	96	196, 14%*
Ratio CO_2/O_2	0·14	0·21	0·27	0·38	0·36	0·42	0·47	0·41	0·39	0·44	Average
O_2 uptake/day	75	95	36	21	22	21	12	7	7	4	0·33

* Percentage by weight of oxygen.

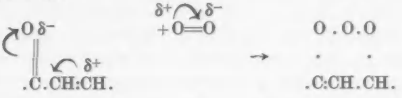
methyl heptyl ketone. These products are, however, of an aldehyde or ketone nature. They represent only 0·6% yield of the acid autoxidized.

DISCUSSION

It has been shown that the $\alpha\beta$-unsaturated keto acids are initial products of autoxidation. Whether these are formed from a hydroperoxide by rearrangement of its oxygen atoms and elimination of water or whether they are slowly formed in an autoxidation process by a reaction which is greatly accelerated by the catalytic effects of certain metals will be considered below. Formed in appreciable amounts at temperatures of about 50°, these acids decrease in concentration as the temperature of the process is raised, to be replaced by their coloured, resinous polymers. At all temperatures these acids give rise to an appreciable proportion of their apparent dimerides. Together they constitute over 90% of the products of autoxidation for the lower-temperature processes. It has been shown also that the molar oxygen uptake by elaidic (or oleic) acid is about 4 atoms at that stage when unchanged acid disappears. Even at lower temperatures it has been shown that about 30% of this uptake is evolved as carbon dioxide and water and that on continuing the autoxidation beyond this stage the oxygen evolved as these products may be 60% of any further uptake. It has been shown, further, that these unsaturated keto acids are peroxide-forming. So also are their supposed dimerides which apparently contain the unsaturated ketone grouping, but in a more stable form than that found in the initial products; a comparative stability which has been explained to be due to the presence of the double bond in a ring formation. These probable dimerides resist alkaline hydrolysis, but when recovered after saponification of the products of an autoxidation they show an oxygen uptake and then give a peroxide reaction.

After the source of heat of an autoxidation process is removed and the reaction chamber has assumed room temperature, there continues a slow oxygen uptake, and the peroxide value of the products shows an increase as though these peroxides were more stable at the lower temperature while continuing to be formed. The author (Ellis, 1950) has shown that a pure unsaturated keto acid exposed to air at room temperatures is distinctly peroxide-forming. Investigating substances of lower molecular weight, Eccott & Linstead (1930) observed that one of the butylideneacetones exploded after keeping some time at ordinary temperatures. In addition to the formation of a peroxide the author's pure product referred to gave an epoxy acid and, in addition to apparent β-ketonic acid formation, an evolution of carbon dioxide was observed. The unsaturated keto acids therefore give peroxides and peroxidation products. Elaidic acid epoxide has been prepared by Pigulevski & Petrova (1926), and by subsequent workers, as a product of peroxide reagents on the acid. So also, from the work of Dakin (1908), β-ketonic acid and carbon dioxide formation can be attributed to peroxide action.

In seeking for the nature of the peroxide responsible for these peroxidation products formed in the autoxidations studied, it would appear that it results from oxygen addition to the unsaturated keto acids. It does not seem reasonable to offer any peroxide structure as forming part of an autoxidation process unless the reduced form is clearly shown to accumulate in the autoxidation products or unless that form is able to resume the peroxide structure in the presence of oxygen gas. The peroxide formed must be very unstable, even at room temperatures, at any rate when in the presence of the oxidizable substrate. It is as though little more than an adsorption of the oxygen takes place. Such an adsorption might be due to electrostatic attraction, but a reaction involving movement of the double bond of the unsaturated ketone system and addition of the oxygen molecule to the β-carbon and to the carbonyl oxygen is suggested in accordance with the equation:



Such a structure would afford a peroxide with two available oxygen atoms and its reduced form would be the original unsaturated keto acid. It has been shown that the disappearance of any appreciable concentration of the unsaturated keto acids as a consequence of the prolonged autoxidation is contemporary with a nearly complete cessation of the reaction.

The above salient findings of this work seem to suggest a simple explanation for the autoxidation process as applied to the mon-ene fatty acids. It would appear that nearly the whole, if not the whole, of the unsaturated acid forms a mixture of the η- and κ-keto-oleic or elaidic acids. These form unstable and therefore active peroxides which give rise, by peroxidation, to the epoxy derivatives and their monoacyl esters, to the scission acids, to β-oxidation and to the other signs of chain oxidation and degradation such as the evolution of carbon dioxide and water. The supposed dimerides would represent a secondary reaction of the unsaturated keto acids, as would the formation of the coloured polymers caused by the action of heat.

In recent years, however, other workers have demonstrated the formation of hydroperoxides in autoxidation processes. It is in the same η- and κ-positions of the carbonyl groups found in this work that Farmer & Sutton (1943) have found hydro-

peroxide groups as a result of the autoxidation of the methyl esters of oleic and of elaidic acids under the influence of ultraviolet light. The same hydroperoxide groups in the same positions have been observed by Atherton & Hilditch (1944), who showed that by oxidation with potassium permanganate their preparations yielded suberic and heptane-1-carboxylic acids, indicating the η- and κ-positions respectively.

The substitution of a hydroperoxide group for one of the hydrogens in a methylene group adjacent to a double bond was previously noted as a result of a similar autoxidation of *cyclo*hexene by Criegee, Pilz & Flygare (1939). The same product was also prepared by Farmer & Sundralingam (1942) by autoxidation in the presence of ultraviolet light at a temperature not exceeding 40°. No recognizable amount of a cyclic ketone was formed, but Dupont (1936*a*, *b*) had identified a 6-keto derivative of 1-methyl*cyclo*hexene by autoxidation at 95° in the absence of a catalyst, and at 70° from 1:2-dimethyl-*cyclo*hexene a 3-keto product was obtained. Also, Cook (1938) obtained the 1-methyl*cyclo*hexen-1-one at 70° in the presence of the catalyst ferrous phthalocyanine. Farmer & Sundralingam (1942) drew attention to these ketone formations at 70° or over, as compared with their own observations that no ketone groups, but those of a hydroperoxide, were formed in the same positions at temperatures below 40°. They observed that the catalyst employed by Cook produced active decomposition of their hydroperoxides and that ketones could be recognized in the products formed. It should be noted, however, that catalysts are not necessary to the autoxidation process, although they accelerate the initial reaction.

The hydroperoxide groups as introduced into the unsaturated fatty acids by Farmer & Sutton (1943) were found to be remarkably heat-stable. No ketones accompanied their formation, nor could ketones be obtained from them by the action of heat. They did not oxidize their substrate. The methyl esters of the fatty acids containing these hydroperoxides, in excess of the unchanged esters, could be concentrated by distillation of the latter at 65°. This is a temperature within the higher range employed in the autoxidation processes of this work.

It is difficult, therefore, to regard the hydroperoxides as precursors of the unsaturated keto acids found in this work. It is still more difficult to regard these hydroperoxides as the oxygen donors to the oxidizable substrate giving rise to the peroxidation products.

There is, on the other hand, little difficulty in fitting all the observations made in this work into a sequence of reactions which go to form the autoxidation processes as a whole. In the first place there are slow formations of the η- and κ-keto groups. These formations are greatly accelerated by the presence of the well-known polyvalent metallic catalysts, but in the absence of these they are so slow as to constitute the so-called 'induction period'. In the second place the $\alpha\beta$-unsaturated ketone systems resulting, take up molecular oxygen to form unstable, and therefore active, peroxides which accelerate the formation of further unsaturated keto acids and so account for the recognized autocatalytic action of a non-catalysed drying oil. At the lower temperatures employed these unsaturated keto acids accumulate and give rise to dimerides, these together constituting perhaps 90 % of the oily products of autoxidation. At the higher temperatures they polymerize giving coloured oils and resins which may then form the greater proportion of the autoxidation products. In the third place the peroxides of the unsaturated ketone groupings, at all temperatures employed, give rise to the peroxidation products, which include the epoxides, the acid scission products (suberic, heptane-1-carboxylic, etc., formed more at the higher temperatures), together with β-ketonic acids, carbon dioxide and water, constituting chain degradation products and representing over 60 % of the reaction of the later stages of autoxidation.

SUMMARY

1. Further evidence has been obtained for the conclusion arrived at in the earlier parts of this work that an oxygen uptake of over 20 % is required in the autoxidation process before there is a marked falling off of the initial rate. A near observed percentage is 22·7, corresponding to an uptake of 4 atoms/mol. of the acids (elaidic or oleic).

2. *Autoxidation at about* 100° yields the following products. (i) A small proportion of the epoxy acid and an appreciable proportion of the monoacyl esters derived from it, the scission products suberic, azelaic, heptane- and octane-1-carboxylic acids. (ii) A predominating proportion of the coloured, resinous polymers of the $\alpha\beta$-unsaturated keto acids, which latter are, as a consequence, reduced to a small yield. There is also present an appreciable proportion of an apparent mixture of isomeric dimerides of the $\alpha\beta$-unsaturated keto acids.

3. *Autoxidation at about* 65° yields the same products, but the coloured resinous polymers are less and the supposed dimerides are more. There is now present nearly 20 % of the epoxy acid (Part 2, Ellis, 1936) and about another 15 % of this acid as monoacyl esters. There are now present significant amounts of the $\alpha\beta$-unsaturated keto acids.

4. *Autoxidation at about* 50° yields the $\alpha\beta$-unsaturated keto acids (*cis*- or *trans*-heptadec-8-en-7-one-1-carboxylic and *cis*- or *trans*-heptadec-8-en-10-one-1-carboxylic acids). Their supposed dimerides will be present in a larger or smaller proportion, but

together they may represent over 90% of the products. There is present about 5% of the epoxy acid, but little or no monoacyl esters and little or no coloured, resinous oils and the acids forming the scission products also appear to be absent or nearly so.

5. *At all the above temperatures* the products give evidence for the presence of β-ketonic acids and considerable volumes of carbon dioxide and water vapour are evolved. This apparent chain oxidation and degradation is regarded as being brought about by peroxides present in small amounts and formed from the unsaturated keto acids which are the initial products whose formation is probably catalysed by certain metals.

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Studies on the more Soluble Complexes of Soil Organic Matter

2. THE COMPOSITION OF THE SOLUBLE POLYSACCHARIDE FRACTION

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In the first paper of this series (Forsyth, 1947*a*) a method of fractionation of the soluble complexes of soil organic matter was presented and the observation made that polysaccharide fractions containing uronic acid groups could be readily isolated in reasonable yield from diverse types of soil. Because of the important effect that such polysaccharides might well have on the physical properties (Martin, 1946) and the biochemical properties (Forsyth, 1948*a*) of the soil, their composition has been studied and to some extent elucidated.

EXPERIMENTAL

Purification of the polysaccharide complex

The polysaccharide fraction was isolated by the selective adsorption procedure previously described (Forsyth, 1947*a*). It was first purified by precipitation into 4 vol. of ethanol containing 2% (v/v) acetic acid followed by further purification by the addition of excess $CuSO_4$ to an alkaline solution of the gum. The precipitated copper complex after washing with water was decomposed with ethanol containing HCl and washed free from Cu^{++} and Cl^- with ethanol. The polysaccharide remained as a white powder which, after a further ethanol precipitation, could be dried by washing with ethanol followed by ether to give an ash-free powder readily soluble in water. Polysaccharide complexes were prepared in this manner from the heath, pine-forest, and agricultural soils previously described (Forsyth, 1947*b*), and also from deciduous woodland and permanent pasture soils, all from north-east Scotland. A similar fraction was prepared from a garden soil in Trinidad, British West Indies. All the preparations from the diverse sources had a slight negative rotation $[\alpha]_D^{20°}$ $-2°$ to $-10°$ in water (*c*, 1·0, as Na salt) and a nitrogen content of 0·1–0·5%. However, on titration with 0·02 N-NaOH equivalents were obtained ranging from 1000 to 1300.

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Qualitative identification of the constituent sugars

The polysaccharides from all sources gave the same sugars on hydrolysis. The sugars were identified by paper chromatography (Partridge, 1946, 1948), confirmation being obtained by spraying papers with phenolic reagents (Forsyth, 1948*b*; Partridge, 1948). It was found advisable because of the complexity of the hydrolysate to chromatograph with four different solvents on one-dimensional chromatograms. Two-dimensional chromatograms were not very helpful with the sugar mixture present. The solvents used were water-saturated *n*-butanol, phenol, *s*-collidine, and amyl alcohol. Although the relative movements of the sugars with respect to the solvent boundary (R_F value) varied to some extent, it was found that their relative movement with respect to one another was remarkably constant in spite of variations in apparatus, temperature, etc. Xylose is the most readily identified of the sugars present in the polysaccharides. The rate of movement of the sugars is therefore expressed with reference to the rate of movement of xylose on the same paper. The factor used (R_X) is the ratio of the distance moved by a sugar to the distance moved by xylose. R_X is much more readily determined than R_F when the spots are very slow moving, as in butanol or amyl alcohol, and may require up to 1 week for full development, by which time the solvent boundary has moved off the paper.

The polysaccharides (5–10 mg.) in N-H_2SO_4 (0·5 ml.) were heated in a sealed capillary tube in a boiling water bath for 5 hr. The seal was then broken, the hydrolysate carefully neutralized with $BaCO_3$ to congo red paper and the precipitate centrifuged down while still in the capillary. The neutral supernatant liquor was then spotted on many chromatogram strips for development with different solvents and spraying with various reagents after development. The polysaccharides from the different soils gave qualitatively identical chromatograms. The sugars identified are shown in Table 1. Spraying with resorcinol, etc. (Forsyth, 1948*b*), showed that ketoses were absent, and spraying with acetylacetone and *p*-dimethylaminobenzaldehyde (Partridge, 1948), demonstrated that no hexosamines were present. Fucose, lyxose, rhamnose, and deoxyribose were chromatographed alongside the hydrolysates and shown to be absent. Galactose, glucose, mannose, arabinose, xylose, and ribose spots were identified. Spots correspond-

Table 1. *One-dimensional chromatograms of polysaccharide hydrolysate and reference sugars*

Spot no.	R_X of spot	Reference sugars	R_X of reference sugars	Phenolic spray
		n-Butanol		
1	0·50	Galactose	0·50	Hexose
2	0·58	Glucose	0·58	Hexose
3	0·83	Mannose	0·80	Pentose
		Arabinose	0·85	Pentose
4	1·00	Xylose	1·00	Pentose
5	1·50	Ribose	1·50	Pentose
		Amyl alcohol		
1	0·31	Galactose	0·29	Hexose
2	0·38	Glucose	0·38	Hexose
3	0·66	Mannose	0·65	Hexose
4	0·81	Arabinose	0·81	Pentose
5	1·00	Xylose	1·00	Pentose
6	1·30	Ribose	1·31	Pentose
		s-Collidine		
1	0·68	Galactose	0·68	—
2	0·78	Glucose	0·78	—
3	0·88	Mannose	0·92	—
		Arabinose	0·86	—
4	1·00	Xylose	1·00	—
5	1·20	Ribose	1·12	—
		Phenol		
1	0·87	Glucose	0·89	—
2	1·00	Galactose	1·00	—
		Mannose	1·02	—
		Xylose	1·00	—
3	1·20	Arabinose	1·23	—
4	1·36	Ribose	1·34	—

ing to uronic residues were invariably found close to the starting line. Moreover, faint traces of glucurone were identified (R_X in collidine, 1·44; in amyl alcohol, 1·90).

Quantitative estimation of the constituent sugars

For the methods used see Flood, Hirst & Jones (1947, 1949). For comparison of the polysaccharides from different sources an internal sugar standard was not used but only the ratios of the sugars present determined. About 10 mg. of each polysacharide was hydrolysed and neutralized as in the qualitative examination. The sugar solution was spotted across a 5 in. chromatogram strip and, after developing, the individual sugars were extracted and estimated by Somogyi's micro sugar reagent (Somogyi, 1945) as described by Flood *et al.* (1947). Unfortunately, it was not realized when the estimations were made that both mannose and arabinose were present and butanol was used as the solvent. The mannose and arabinose were therefore estimated together and the molar ratios for this spot are calculated in Table 2 by division by 165, the mean of a hexose and pentose molecule. It is probable that the mannose was generally present in greater amount than arabinose.

Hydrolysis with N*-sulphuric acid*

The polysaccharide complex (2 g.) was heated with N-H_2SO_4 (100 ml.) at 90–95°, the reaction being followed by polarimetric observations. $[\alpha]_D^{20}$ (*c*, 2, in N-H_2SO_4) −5° (initial value); +21° (1 hr.); +43° (2 hr.); +45° (3 hr.); +45° (5 hr.). Iodometric titration of samples after 5 hr. gave a value for reducing sugars corresponding to about 80% of the original polysaccharide. The cooled hydrolysate was neutralized with $BaCO_3$ and filtered. The neutral solution was concentrated under reduced pressure to a small volume and poured into methanol. The precipitated Ba salts were collected (0·8 g.), washed with methanol and dried. Concentration of the methanol filtrate gave a sugar syrup (1·3 g.).

(*a*) *The sugars.* The syrup was dissolved in water (25 ml.) and volumes of the order of 2–3 μl. spotted on to paper strips along with reference sugars as before. Galactose, glucose, mannose, arabinose, xylose and ribose were again detected. Neither ketoses, hexosamines, rhamnose, fucose, lyxose nor deoxyribose were present. To 10 ml. of the sugar solution the phenylhydrazine reagent of Hirst, Jones & Woods (1947) was added and the mannose estimated as phenylhydrazone (found, 15·2% mannose). The remainder of the sugar solution was used for the estimation of xylose by the method of Breddy & Jones (1945) as modified by Wise & Ratliff (1947) (found, 16·5% xylose).

Table 2. *Sugar ratios in the polysaccharide complexes from diverse soils*

(*A*, Amount of sugar estimated (μg.); *B*, molar ratio.)

	Heath soil		Pine forest soil		Deciduous woodland soil		Permanent pasture soil		Trinidad soil	
Sugars	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Galactose	121	1·8	370	1·9	256	1·8	314	1·8	291	1·9
Glucose	139	2	385	2	281	2	360	2	314	2
Mannose, arabinose	225	3·5	474	2·6	462	3·6	457	2·8	381	2·7
Xylose	197	3·4	619	3·9	434	3·1	421	2·8	392	3·0
Ribose	25	—	24	—	76	—	49	—	15	—

Larger scale investigation

The work described below was carried out entirely on the polysaccharide complex prepared in the usual manner from a large sample of a humus-rich agricultural soil. About 10 g. of the purified soil polysaccharide was prepared from 10 kg. of air-dried soil containing 8·5% organic matter. The air-dried polysaccharide contained 10–20% water. It did not reduce Fehling solution and had a slight negative rotation $[\alpha]_D^{20°}$ −5° in water (*c*, 1·0, as Na salt). After dialysis for 1 week against distilled water the polysaccharide had a titration equivalent of 1185 (0·02 N-NaOH). (Found: OMe, nil; N, 0·34; furfural, 18·1, estimated as phloroglucide after treatment of the polysaccharide with boiling 12% (w/v) HCl in the usual way; methylfurfural, nil; hexosamine, nil; uronic anhydride, 15·8%, calc. from the CO_2 liberated on boiling with 12% (w/v) HCl.) A substance containing 15·8% uronic anhydride and no other acidic residues should have an equiv. wt. of 1114 (found by titration, 1185). This proportion of uronic anhydride accounts for 3·5% furfural, leaving 14·6% furfural contributed by the pentosans. Therefore the calculated pentosan—assuming xylose and arabinose to be present in the ratio 3:1 (*vide infra*)—is 23·8% (found 24·7%, by chromatographic estimation).

(*b*) *Chromatography on a cellulose column.* Using the technique introduced by Hough, Jones & Wadman (1948), a column of Whatman no. 1 cellulose, 20 × 2 cm., was prepared by breaking up the filter paper in water-saturated collidine in a Waring blender and transferring to the column with gentle suction. The sugar syrup from a hydrolysate of 1 g. of polysaccharide, prepared as before, was added to the top of the column which was then eluted with water-saturated collidine under slight pressure at the rate of 10 ml./hr. Each 10 ml. portion was collected separately, concentrated to about 1 ml. and spotted on to paper-strip chromatograms using the four solvents and also phenolic sprays. Because of the partial resolution of the complex mixture the identification of the constituent sugars was more precise and the absence of other sugars definitely confirmed. The volumes of the fractions and the sugars identified in them were as follows: first 200 ml., nil; 60 ml., ribose; 70 ml., ribose + xylose; 20 ml., xylose; 150 ml., xylose + mannose + arabinose; 20 ml., mannose + arabinose + glucose; 60 ml., mannose + arabinose + glucose + galactose; 170 ml., glucose + galactose; 30 ml., galactose; 20 ml., nil. In the last two sugar fractions glucose could be identified

as the osazone and galactose as the phenylmethylhydrazone, since no interfering sugars such as mannose or arabinose were present. All the fractions containing arabinose (less than half the sugars) were combined and evaporated to a syrup which was taken up in 2 ml. of water, and the arabinose was estimated as the benzoylhydrazone (Hirst *et al.* 1947). No interfering sugars were present in these fractions (found 5·9% arabinose).

(*c*) *Quantitative paper chromatography.* Polysaccharide (10·205 mg., in 0·5 ml. of N-H_2SO_4) was heated in a sealed capillary in a boiling water bath for 5 hr. The seal was broken and rhamnose (3·120 mg.) weighed in as internal standard. The hydrolysate was then carefully neutralized with $BaCO_3$ and centrifuged while still in the capillary. The supernatant liquor was spotted on 'quantitative' strips and developed with amyl alcohol for 1 week. The results are shown in Table 3.

(*e*) *Composition of soil polysaccharide.* The results indicate (Tables 3 and 4) that the soil polysaccharide complex contains the following sugars in the proportions indicated: D-galactose (2 mol.); D-glucose (2 mol.); D-mannose (2 mol.); L-arabinose (1 mol.); D-xylose (3 mol.) and 2 mol. of an aldobionic acid containing D-glucose (1 mol.) and glucuronic acid (1 mol.).

Oxidation of the polysaccharide with potassium metaperiodate

(*a*) Soil gum (39·346 mg.) was weighed into a dry steamed 100 ml. stoppered bottle, dissolved in 10 ml. of distilled water and neutralized. 0·3 M-sodium metaperiodate (5 ml.) was then added, followed by KCl (2·5 g.) and water to 40 ml. The bottle was then shaken for 9 days in diffuse light, 5 ml. samples being withdrawn on the seventh, eighth and ninth days. Excess ethylene glycol was added to destroy the residual periodate and the formic acid produced was titrated with 0·01 N-NaOH using methyl red as indicator. The reaction was found to be complete by the seventh day showing that uronic acid was not present as an end group (Brown, Dunstan, Halsall, Hirst & Jones, 1945):

39·346 mg. polysaccharide ≡ 7·45 ml. 0·01 N-NaOH,
hence 522 g. polysaccharide ≡ 1 mol. formic acid.

(*b*) In another set of identical bottles the whole 40 ml. was titrated after the eighth day for excess periodate using standard sodium arsenite and I_2. From the results it was calculated that the 522 g. of polysaccharide which liberated 1 mol. of formic acid consumed 3·58 mol. of periodate.

(*c*) About 50 mg. of the polysaccharide were oxidized as in (*a*). After the excess periodate had been removed with ethylene glycol the solution was dialysed till free from salts. The polymeric aldehyde was then precipitated with ethanol, filtered, washed with ethanol and then ether and dried. About 20 mg. were hydrolysed in a sealed capillary tube on a boiling water bath with 0·5 ml. 2 N-H_2SO_4 for 5 hr. The neutralized solution was chromatographed and only glucose and glucuronic acid could be detected.

Table 3. *Quantitative analysis of hydrolysate of soil polysaccharide complex*

	Sugar estimated from paper (μg.)	Sugar present in hydrolysate (mg.)	Hexosan or pentosan (%)	Approx. molar ratio
Rhamnose*	280	3·120	—	—
Polysaccharide	—	10·205	100	—
Galactose	136	1·51	13·3	2
Glucose	140	1·56	13·8	2
Mannose	147	1·64	14·5	2
Arabinose	82	0·91	7·8	1
Xylose	163	1·82	15·7	3
Ribose	12	0·14	1·2	—

* International standard.

(*d*) *Barium salts.* The precipitated Ba salts (0·8 g.) from the hydrolysis with N-H_2SO_4 had $[\alpha_2]_D^{20°}$ +57° in water (*c*, 0·3, as Ba salt). (Found: Ba, 17·1 (estimated as $BaSO_4$); furfural, 8·9%. The Ba salt of a hexose aldobionic acid requires furfural, 10·0; Ba, 16·2%.) The Ba salt (10 mg.) was hydrolysed in a sealed tube in a water bath with 2 N-H_2SO_4 (0·5 ml.) for 7 hr., giving glucose and glucuronic acid (or glucurone, depending on the solvent) as shown by chromatography. Quantitative chromatography confirmed that the acid and the sugar were present in equimolar quantities.

Table 4. *Calculated and observed values for soil polysaccharide*

(Calculated values are based on the molecular proportions of sugars given below.)

	Calculated (%)	Observed (%)
Galactan	14·8	13·3*
Glucosan	14·8	13·8*
Mannan	14·8	14·5* / 15·2†
Araban	6·1	7·8* / 5·9‡
Xylan	18·2	15·7* / 16·5§
Uronic anhydride	16·2	15·8
Furfural	18·5	18·1
Equivalent	1088	1185

* By paper chromatography. † As phenylhydrazone.
‡ As benzoylhydrazone.
§ As dibenzylidene dimethylacetal.

Autohydrolysis of soil polysaccharide

Aqueous solutions of the polysaccharide are sufficiently acid to bring about a partial hydrolysis of the polysaccharide when heated in a boiling water bath for 24 hr. Soil polysaccharide (2 g.) was heated with water (30 ml.) on a boiling water bath for 24 hr., by which time the rotation had become constant ($[\alpha]_D^{20°}$ +24°) and the iodometric titration of samples gave a value for reducing sugars corre-

sponding to about 15% of the original gum. The cooled solution when poured into ethanol (100 ml.) gave an alcohol-insoluble polysaccharide *B* (1·7 g.), which was washed with ethanol and then ether and dried. The filtrate from *B* was concentrated under reduced pressure to a syrup. Paper chromatography showed that arabinose was present in quantity. Considerable amounts of ribose had also been produced by hydrolysis and traces of galactose and xylose were detected. No glucose, mannose or uronic acid had been liberated. The syrup was made up to 2 ml. with water, and arabinose was estimated as the benzoylhydrazone by the method of Hirst *et al.* (1947). (Found, 6·7; calc., 6·1%, if all the arabinose were liberated.)

Polysaccharide B

Polysaccharide *B* was a white powder easily soluble in water; $[\alpha]_D^{20°}+20°$ in water (*c*, 1). The equiv. wt. (by titration with 0·02 N-alkali) was 974. Furfural, 16·2% (estimated as phloroglucide after boiling with 12% (w/v) HCl under the standard conditions). Uronic anhydride, 17·9% (calc. from the CO_2 liberated on boiling with 12% (w/v) HCl). (A substance containing 17·9% uronic anhydride with no other acidic residue should have an equivalent weight of 983; found by titration, 974. This proportion of uronic acid accounts for about 4% of the furfural, leaving 12·2% furfural contributed by the pentosan.) Polysaccharide *B* (12·394 mg. in 0·5 ml. of N-H_2SO_4) was heated in a sealed capillary in a boiling water bath for 5 hr. The seal was broken and rhamnose (4·316 mg.) weighed in as reference sugar. The hydrolysate was neutralized and chromatographed and the sugars estimated as before. No arabinose or ribose were detected. The aldobionic acid fraction was present as before. For analysis, see Table 5.

Table 5. *Calculated and observed values for polysaccharide B*

(Calculated values are for a polysaccharide containing galactose (2 mol.); glucose (2 mol.); mannose (2 mol.); xylose (3 mol.) and aldobionic acid (2 mol.; each consisting of glucose and glucuronic acid).)

	Calculated (%)	Observed (%)
Galactan	15·8	13·7
Glucosan	15·8	16·0
Mannan	15·8	15·4
Xylan	19·4	18·3 18·9*
Uronic anhydride	17·2	17·9
Furfural	16·3	16·2
Equivalent	983	974

* From furfural estimation.

Polysaccharide *B* was also oxidized with potassium metaperiodate, and the formic acid produced and the periodate consumed estimated as before. 430 g. of *B* liberated 1 mol. of formic acid and consumed 2·84 mol. of periodate. The polymeric aldehyde produced again gave only glucose and glucuronic acid on hydrolysis. Once again the uronic acids were not end groups.

DISCUSSION

A striking feature of the results is that all the polysaccharide preparations, from several diverse types of soils, have very similar properties. Although differences in optical rotations and titration equivalents were noted, all the polysaccharides gave the same sugars on hydrolysis. The sugars identified were galactose, glucose, mannose, arabinose, xylose, and a uronic acid fraction containing glucuronic acid. Ribose was also invariably present in small amount. Estimation of the sugars present in the polysaccharides showed that the sugars, especially the pentoses, varied to some extent in the different samples, but it is considered that the unexpected similarity is much more striking than the minor discrepancies noticed and is a remarkable reflexion of the uniformity in total carbohydrate metabolism which must exist in widely different soil types (Forsyth, 1948*a*).

The degree of homogeneity of the polysaccharide complex from any one soil is not known. Although all attempts to separate significantly different fractions by partial ethanol and acetone precipitation were unsuccessful, this is no proof of homogeneity, since the complex polysaccharides are particularly difficult to separate. The presence of traces of ribose and nitrogen in the gums suggests that they have not been completely freed from ribonucleic acid. This is frequently the case with polysaccharides synthesized by soil bacteria (Forsyth & Webley, 1949) and strengthens the hypothesis that the polysaccharide complex is a synthetic microbial product. Polysaccharide mucilages approaching the degree of complexity of the soil polysaccharide complexes have been isolated from soil bacteria growing in artificial media (Forsyth & Webley, 1949). The highly selective method of isolation (adsorption at pH 3 and elution at pH 6–7 on charcoal) might be thought to lead to a fairly homogeneous product. Neutral polysaccharides such as bacterial levans and dextrans, when added to the soil extract and treated under the isolation conditions used, are not adsorbed by the charcoal and therefore do not contaminate the soil polysaccharide complex.

Even although the degree of homogeneity of the polysaccharide fraction is not proved, and absolute homogeneity may be considered unlikely, the same complex can be isolated from a soil repeatedly, and similar complexes from a very wide variety of soils. The complex is therefore a characteristic fraction of soil organic matter, and as such was isolated in a relatively large amount from a rich agricultural soil and further investigated. It was hydrolysed and the constituent sugars identified and estimated both by chromatographic analysis and with specific precipitants. The results indicate that this soil polysaccharide complex contains the following sugars in

the proportions indicated: D-galactose (2 mol.); D-glucose (2 mol.); D-mannose (2 mol.); L-arabinose (1 mol.); D-xylose (3 mol.); and an aldobionic acid containing D-glucose (1 mol.) and glucuronic acid (1 mol.). Considering the equivalent weight and other quantities determined, the polysaccharide has therefore a repeating unit which may be represented by the following symbols:

$$Ga_2:G_2:M_2:A_1:X_3\,(G:U)_2,$$

where Ga = D-galactose, A = L-arabinose, G = D-glucose, X = D-xylose, M = D-mannose and U = D-glucuronic acid. By estimating the formic acid produced and the periodate consumed on oxidation of the polysaccharide with potassium metaperiodate (Brown *et al.*, 1945; Halsall, Hirst & Jones, 1947), it was found that the above repeating unit liberates 4 mol. of formic acid with the consumption of 14–15 mol. of periodate. Since, under the mild conditions of oxidation used, there is no continual rise in formic acid titre followed by liberation of iodine, the glucuronic acid residues are not end groups. Examination of the polymeric aldehyde produced by periodate oxidation showed that glucose and glucuronic acid were the only sugars not attacked and therefore are the only sugar residues not containing two adjacent hydroxyl groups.

The slightly acidic gum undergoes autohydrolysis when its aqueous solution is heated, the products being arabinose, ribose, traces of galactose and xylose, and a polysaccharide *B* composed of galactose, glucose, mannose, xylose and an aldobionic acid in the approximate molar ratios 2:2:2:3:2. All the arabinose residues are removed under conditions which indicate that they are attached to the polysaccharide by furanoside links. The traces of galactose and xylose found are probably derived from 'overhydrolysis' of polysaccharide *B*. By estimating the formic acid produced and periodate consumed during metaperiodate oxidation of *B* it appears that the repeating unit of *B* required 13–14 mol. of periodate and produced 4·7 mol. of formic acid. Glucose and glucuronic acid were again the only sugars not oxidized.

SUMMARY

1. Ash-free polysaccharide complexes have been obtained from widely different pedological, vegetational and climatic types of soil.

2. All the polysaccharide preparations have very similar properties. They all contain the same sugars, galactose, glucose, mannose, arabinose, xylose, and glucuronic acid, although the proportions, especially of the pentoses, vary to some extent in the different samples.

3. They all contain small amounts of nitrogen and ribose, presumably as ribonucleic acid, suggestive of a microbial origin.

4. From a humus-rich agricultural soil a polysaccharide complex has been isolated in relatively large quantity and the constituent sugars determined both by paper chromatography and by specific precipitants. The results agree well with a repeating unit containing galactose (2 mol.); glucose (2 mol.); mannose (2 mol.); arabinose (1 mol.); xylose (3 mol.); 2 mol. of an aldobionic acid consisting of glucose and glucuronic acid.

5. Mild hydrolysis of the polysaccharide gives an arabinose-free polysaccharide containing galactose (2 mol.); glucose (2 mol.); mannose (2 mol.); xylose (3 mol.); the aldobionic fraction as before.

6. The oxidation of both the original and the degraded polysaccharides with potassium metaperiodate has been studied.

I wish to thank Prof. E. L. Hirst, F.R.S., and Dr J. K. N. Jones for their interest and advice. I also wish to thank Prof. Hirst and the University authorities for making available to me the facilities of the Chemistry Department, Manchester University, where much of the above experimental work was carried out.

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The Porphyrins of Acute Porphyria. The Detection of hitherto Unrecognized Porphyrins

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Waldenström (1934) reported the natural occurrence in acute porphyria of a uroporphyrin III, but Watson, Schwartz & Hawkinson (1945) and Prunty (1946) found evidence for the belief that this Waldenström porphyrin was a mixture of a large proportion of uroporphyrin I with a smaller amount of a type III porphyrin. The observations of Watson *et al.* (1945) also indicated the presence in the urine of porphyrin other than coproporphyrin and the uroporphyrin 'mixture'. The mechanism involved in the porphyrin metabolism of acute porphyria must be regarded as being of great importance, and a necessary step in the elucidation of this mechanism is the fullest possible characterization of the porphyrins present in this disease. Material from two new cases of acute porphyria observed in St Thomas's Hospital forms the basis of the present study on the nature of these porphyrins. The electrolyte metabolism in case 1 is the subject of a separate report (Prunty, 1949).

EXPERIMENTAL

Quantitative methods. The methods previously used for the determination of urinary and faecal coproporphyrin and faecal uroporphyrin (Prunty, 1946) were employed. The urinary coproporphyrin extracts in 0·2% (w/v) HCl were given a final washing with $CHCl_3$ before estimation in a modified Hilger fluorimeter. Urinary total porphyrin was estimated by the calcium phosphate adsorption technique

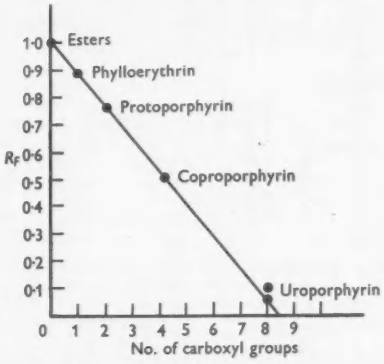

Fig. 1. Paper partition chromatography of known porphyrins. Relation of R_F values to number of carboxyl groups in porphyrins.

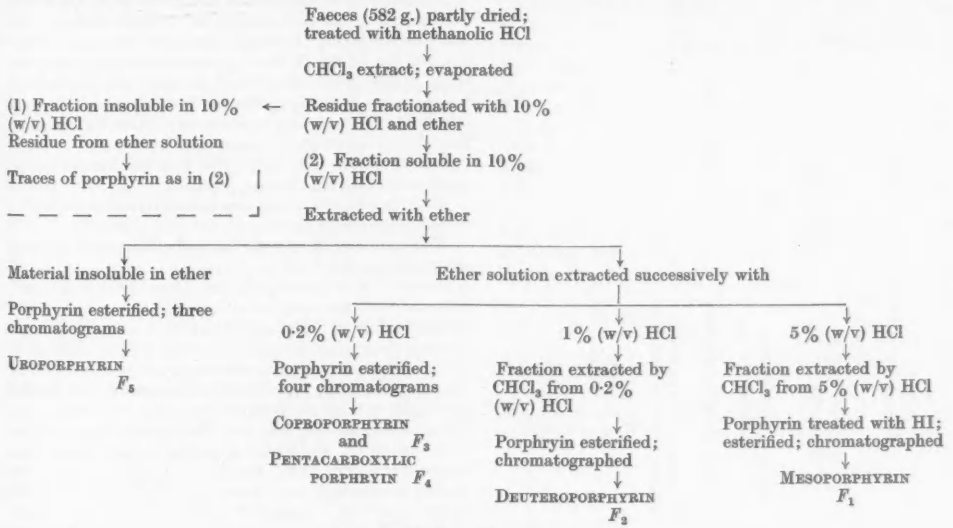
Faeces (582 g.) partly dried; treated with methanolic HCl
$CHCl_3$ extract; evaporated
Residue fractionated with 10% (w/v) HCl and ether
(1) Fraction insoluble in 10% (w/v) HCl
Residue from ether solution
Traces of porphyrin as in (2)
(2) Fraction soluble in 10% (w/v) HCl
Extracted with ether
Material insoluble in ether
Porphyrin esterified; three chromatograms
UROPORPHYRIN F_5
Ether solution extracted successively with
0·2% (w/v) HCl
Porphyrin esterified; four chromatograms
COPROPORPHYRIN and F_3
PENTACARBOXYLIC PORPHYRIN F_4
1% (w/v) HCl
Fraction extracted by $CHCl_3$ from 0·2% (w/v) HCl
Porphryin esterified; chromatographed
DEUTEROPORPHYRIN F_2
5% (w/v) HCl
Fraction extracted by $CHCl_3$ from 5% (w/v) HCl
Porphyrin treated with HI; esterified; chromatographed
MESOPORPHYRIN F_1

Fig. 2. Isolation of faecal porphyrins.

Liver (1162 g.) partly dried; moistened with acetic acid and extracted with ether
Ether solution fractionated into HCl
Residue treated with methanolic HCl
Ester mixtures on primary chromatogram
0·2% (w/v) HCl extract from ether
5% (w/v) HCl extract from ether (porphyrin extracted by $CHCl_3$ from 5% (w/v) HCl)
Porphyrin extracted by $CHCl_3$ from this solution
Porphyrin treated with HI; esterified
Esterified; chromatographed
Mixed coproporphyrin +Tricarboxylic porphyrin L_2
Mesoporphyrin L_1
Fraction A
Fraction B
Fraction C
Fraction D
Hydrolysed and extracted with ether; ether fractionated into HCl
Three secondary chromatograms
Two secondary chromatograms
Three secondary chromatograms
0·2% (w/v) HCl extract from ether. (Porphyrin not extracted by $CHCl_3$ from 0·2% (w/v) HCl)
Porphyrin esterified; two chromatograms
Tricarboxylic porphyrin +Coproporphyrin L_3
Uroporphyrin L_4

Fig. 3. Isolation of liver porphyrins.

of Sveinsson, Rimington & Barnes (1949), the final assays being carried out fluorimetrically. This alteration from the method previously used was necessitated by inability to obtain suitable samples of kieselguhr for porphyrin adsorption. Differential estimation of coproporphyrin I and III isomers in mixtures was carried out by a modification (McSwiney & Prunty, unpublished) of the method of Schwartz, Hawkinson, Cohen & Watson (1947). Porphobilinogen was estimated with *p*-dimethylaminobenzaldehyde (Prunty, 1946) and urobilinogen by the method of Watson (1936).

Chromatography of porphyrin esters. Adsorbents employed were 'alumina for adsorption' (Savory & Moore, 'after Brockmann'); Talc B.P. or $CaCO_3$ A.R. Solvents used were redistilled $CHCl_3$ A.R., benzene A.R. and redistilled light petroleum A.R. (b.p. 40–60°).

Partition chromatography of porphyrin mixtures. The technique used was that described by Nicholas & Rimington (1949), employing a lutidine:water system. Preliminary purification was performed as follows: Crude ester fractions were hydrolysed by contact with 7 N-HCl for 48 hr. at room temperature, the porphyrins and accompanying brown pigments were then adsorbed directly on to a column (0·5 × 3 cm.) packed with talc suspended in distilled water. The non-porphyrin pigment was adsorbed on the top of the column with the porphyrins in a band beneath. The column was washed with distilled water until free from inorganic salts and then with N-NH_3 which eluted the non-fluorescent pigments. The porphyrin was eluted with 0·1 ml. 10 N-NH_3 and this solution applied to the paper for partition chromatography. Ester fractions from previous chromatography were dissolved in $CHCl_3$ and a fraction containing approx. 50 μg. ester was evaporated to dryness under reduced pressure and the residue saponified as above. The HCl was removed in a vacuum desiccator over KOH, and the porphyrin hydrochloride dissolved in 0·1 ml. 2 N-NH_3. In order to avoid misinterpretation through slight changes in R_F values owing to variation in conditions, known markers were run on the strips in every case alongside the material under investigation. The results obtained with such markers are depicted in Fig. 1, the R_F values bearing a linear relation to the number of carboxyl groups in the porphyrin.

Conversion to mesoporphyrin. Protoporphyrin was reduced with HI according to Grinstein & Watson (1943) and isolated as mesoporphyrin ester.

Decarboxylation of uroporphyrin. This was carried out as previously described (Prunty, 1946).

Absorption spectra. These were measured with a Hartridge reversion spectroscope.

Melting points. These were observed microscopically on an electrically heated block.

Extraction of urinary porphyrins. Urine (12 l.) from case 1 collected from the 15th to 21st hospital days inclusive was brought to pH 3·4 with 2 N-HCl and 100 g. talc added. After shaking thoroughly the mixture was allowed to stand at 4° for 5 days, when nearly the total porphyrin was adsorbed on the talc. The latter was filtered off, air-dried and treated overnight with 100 ml. methanol saturated with HCl. After filtration and washing with this HCl-methanol mixture and then with $CHCl_3$, the filtrate and washings were poured into excess water. The $CHCl_3$ solution of porphyrin esters was washed successively with water, 10% (w/v) NH_3 and with water, and then evaporated to dryness. The esters were chromatographed on alumina after dissolving in $CHCl_3$ and

adding 7 vol. of light petroleum, the addition of which caused some precipitation. The precipitated porphyrin was filtered off and the filtrate put on the column. The precipitate was then dissolved in $CHCl_3$ and 5 vol. of light petroleum added. The process was repeated with 1:4, 1:3, 1:2·5 and 1:1 $CHCl_3$-light petroleum mixtures. By this means all the esters were introduced on to the column, the chromatogram developed and the successive bands eluted and collected.

Isolation of faecal porphyrins. The flow sheet of this process is illustrated in Fig. 2. Faeces (582 g.) collected from case 1 from 13th to 23rd hospital days inclusive were partially dried on the water bath and treated overnight with methanol saturated with HCl. The methanolic solution contained the porphyrin esters and much fatty material, and these substances were transferred to $CHCl_3$. After washing the latter it was evaporated to dryness. The residue was shaken mechanically with successive portions of 10% (w/v) HCl (total vol. 260 ml.) for 1 hr., and then allowed to stand 2 days with the last portion. The combined HCl solutions were extracted with ether, a black precipitate appearing in the ether layer. The precipitate was filtered off, and repeatedly extracted with 10% (w/v) HCl, the latter being returned to the main portion. The ether was also extracted with a small volume of 10% (w/v) HCl and the latter also added to the main bulk. Sodium acetate was added to the HCl solution of porphyrin esters until the solution was no longer acid to congo red and the ether-soluble porphyrin removed with ether. Ether-insoluble porphyrin precipitated at the interface and after filtration was dissolved in a small vol. of 10% (w/v) NH_3 and returned to the aqueous fraction which was then adjusted to pH 3·4 and treated with talc. The further procedure was identical with that used in the extraction of urine.

Isolation of porphyrins from liver. The liver freshly obtained post mortem from case 2 weighed 1162 g. and was partially dried *in vacuo* over P_2O_5 after mincing. It was moistened with acetic acid and repeatedly extracted with ether in a mechanical shaker and the porphyrins fractionated as described in the flow sheet (Fig. 3). The ether extracts were fractionated into HCl. The residual liver tissue was treated with HCl-methanol. The porphyrin esters thus extracted were chromatographed in the manner described above.

RESULTS

Quantity of porphyrin excreted

Fig. 4 indicates the results obtained for the coproporphyrin and uroporphyrin excretion in case 1. Urinary coproporphyrin reached levels as high as 2·78 mg./day and uroporphyrin 35·2 mg./day. In the faeces there were up to 4·3 mg./day coproporphyrin and 2·0 mg./day of uroporphyrin.

Urinary porphyrins

A sample of the crude porphyrin ester fraction, from case 1, prior to initial chromatography on alumina was subjected to paper chromatography. The free porphyrin mixture was found to contain porphyrins with 4, 5, 6, 7 and 8 carboxyl groups (Table 1). The porphyrins with 6 and 7 carboxyl groups occurred only in traces and the material containing 6 carboxyl groups was lost on subsequent purification (see below).

Table 1. *Partition chromatograms of crude esters from urine*

R_F value	Amount	No. of carboxyl groups	Nature of porphyrin present
0·07	Large	8	Uroporphyrin
0·12*	Trace	—	—
0·18	Trace	7·0	?
0·27	Trace	6·2	?
0·37	Moderately large	5·3	?
0·5	Large	4·0	Coproporphyrin

* Pure samples of uroporphyrin consistently give two spots. It is thought both are due to uroporphyrin (Nicholas & Comfort, 1949). To avoid confusion in subsequent tables the 0·12 spot is omitted.

The primary chromatogram from the porphyrin extraction yielded four main fractions. The first was eluted with $CHCl_3$-light petroleum 1:3 and after re-esterification rechromatographed on alumina from $CHCl_3$-light petroleum 1:9 in which it was soluble. This yielded mainly fraction U_1 eluted with $CHCl_3$-light petroleum 1:5 and traces of fraction U_2 eluted with 1:4 solvent mixture. The second fraction from the primary chromatogram, eluted with 1:2·5 solvent mixture was likewise rechromatographed, after re-esterification, from 1:8 solvent mixture in which it was soluble. This yielded mainly fraction U_2 and some U_1. Fraction U_3 was eluted from the primary chromatogram with 1:1 solvent mixture. Fraction U_4 on the primary chromatogram was 'red brown' in appearance and after elution with $CHCl_3$-acetic acid 5:1 and re-esterification was rechromatographed on alumina and yielded mostly material corresponding to fraction U_3, with small amounts of U_2 and U_1 which were

Table 2. *Characteristics of urinary porphyrins*

Fraction no.	Porphyrin	Ester: Amount (mg.)	Ester: No. of times crystallized	Ester: M.p. (°)	Proportion of $CHCl_3$ to light petroleum used in the eluent from Al_2O_3	Absorption band I ($\lambda_{max.}$; mμ.)	Results from partition chromatogram: R_F value	Results from partition chromatogram: No. of carboxyl groups
U_3	Uroporphyrin	55	2	271–274	1:1	625·6	0·07	8
							+ trace 0·16	7
U_2	?	1	5	222–227	1:4	622·6	0·4	5
							+ trace 0·5	4
U_1	Coproporphyrin I	4·8	5	250–251	1:5	621·4	0·5	4

again rechromatographed on alumina from 1:7 solvent mixture. The respective moities of fractions U_1, U_2 and U_3 were combined and crystallized from $CHCl_3$-methanol (Table 2).

Fraction U_2. 1·0 mg. ester, m.p. 222–227° after five crystallizations, containing mainly a porphyrin with five ester groups, and traces of porphyrin with four (the latter probably coproporphyrin) (Fig. 7).

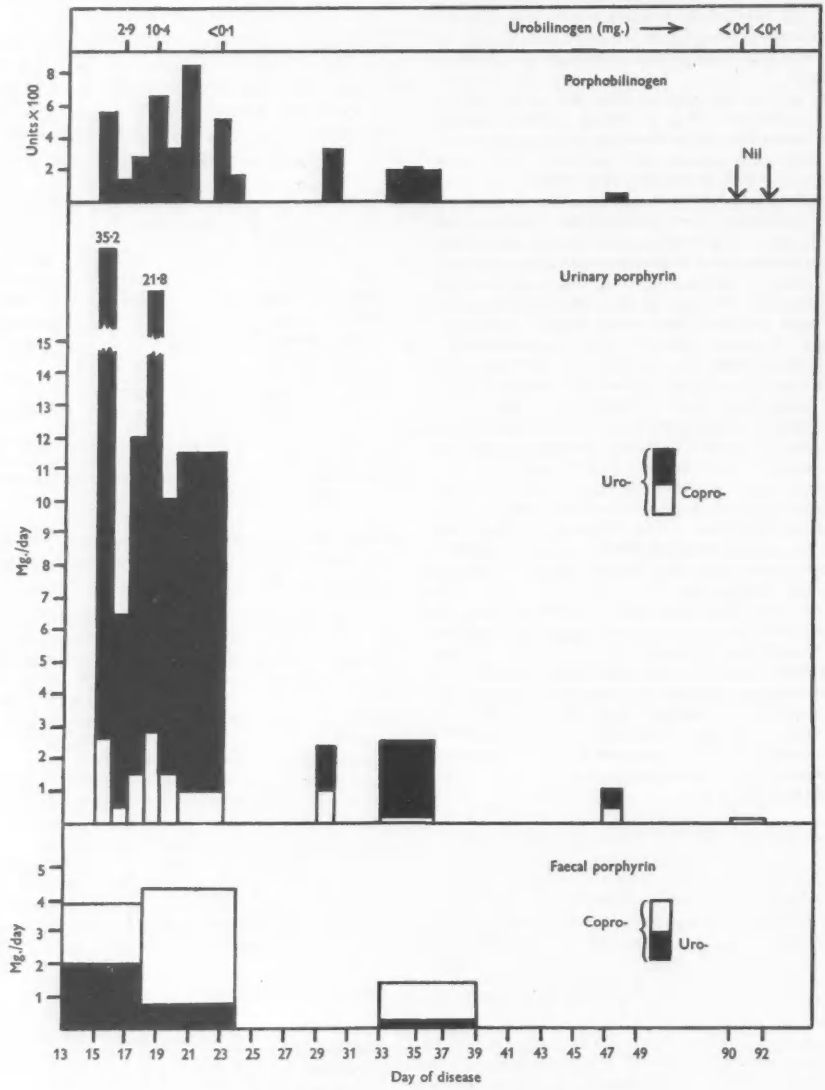

Fig. 4. Daily porphyrin excretion, case 1.

Fraction U_1. This yielded 4·8 mg. ester m.p. 250–251° after five crystallizations and by partition chromatography the material contained only four ester groups. This fraction is coproporphyrin I tetramethyl ester.

Fraction U_3. 55 mg. ester, m.p. 271–274°, after two crystallizations. This was composed mainly of material with eight ester groups plus a trace with seven. A portion of this material was hydrolysed with 10% (w/v) HCl overnight;

the porphyrin could be extracted with ethyl acetate after the pH of the solution had been adjusted for 3·4 with sodium acetate. This fraction corresponds to 'uroporphyrin III'. Attempts to separate this fraction into components on calcium carbonate columns from benzene-light petroleum mixtures, after solution in a minimal amount of $CHCl_3$, were unsuccessful.

Decarboxylation and esterification of 9·1 mg. yielded an ester which was chromatographed on calcium carbonate from benzene-light petroleum 1:1. Benzene-$CHCl_3$ 20:1 eluted the major part of the porphyrin, which was rechromatographed on alumina. A microscopic amount of porphyrin remaining as a second band on the calcium carbonate was eluted with $CHCl_3$, and after three crystallizations had m.p. 224–228°. By partition chromatography it contained four carboxyl groups, but was not further examined. The main bulk of the porphyrin was chromatographed on alumina from $CHCl_3$-light petroleum 1:8 and was eluted with 1:4 mixture. Partition chromatography of the eluted porphyrin showed the presence of only four ester groups (Table 3). After two recrystallizations from $CHCl_3$-methanol this fraction yielded 3·0 mg. coproporphyrin I ester, m.p. 250–252° (Fig. 5). On recrystallization from ether the mother liquor yielded a microscopic quantity of ester of m.p. 140°. The substance did not remelt above 144°, but the form of the crystals strongly resembled that of authentic coproporphyrin III methyl ester (Fig. 6).

An additional 2·7 mg. of the uroporphyrin ester was decarboxylated and the ester obtained chromatographed on alumina from $CHCl_3$-light petroleum. The porphyrin, which appeared homogeneous, was assayed for the relative proportions of coproporphyrins I and III. This assay showed that the mixture contained approximately 75% coproporphyrin I and 25% coproporphyrin III.

Faecal porphyrins

Ether-soluble porphyrins. The ether-soluble porphyrins, from case 1, were fractionated into fractions soluble in 0·2, 1 and 5% (w/v) HCl respectively.

Fraction F_1. The porphyrin soluble in 5% (w/v) HCl was esterified and treated with HI. After esterification and chromatography on alumina it yielded 5·8 mg. mesoporphyrin IX ester, m.p. 216–218°, containing two ester groups (Table 4).

Fraction F_2. The porphyrin soluble in 1% (w/v) HCl yielded after esterification, chromatography on alumina and crystallization from $CHCl_3$-methanol 3·1 mg. deuteroporphyrin IX ester, m.p. 223°, containing two ester groups.

The porphyrin soluble in 0·2% (w/v) HCl was returned to ether, the latter removed and the residue esterified with HCl-methanol. Four chromatograms were run on alumina in the manner described above. Table 5 represents a composite chromatogram of this series.

Fraction F_3. In running the secondary chromatogram there was a marked tendency for this fraction to separate into two bands on the column (Band I*a*, *b*, Table 5). Component *a* weighed 5·5 mg. and after five crystallizations had m.p. 248–250°. Component *b* after five crystallizations yielded 14 mg. ester of m.p. 250–252°. The mother liquor of this yielded from ether after three crystallizations 0·8 mg. ester of m.p. 149°, remelting at 168–171°. Equal parts of ester crystals *a* and the portion of *b* melting at 250–252° were mixed and gave crystals from $CHCl_3$-methanol of m.p. 248–252°. The mother liquor contained only a minute amount of porphyrin. The mixed crystals on chromatography on alumina behaved as an entity. Each of the two batches of ester used for the mixed crystallizations contained four ester groups as shown by partition chromatography.

Table 3. *Properties of decarboxylation products of urinary uroporphyrin*

Porphyrin	M.p. of ester (°)	Absorption band I ($\lambda_{max.}$; mμ.)	Results from partition chromatogram: R_F value	Results from partition chromatogram: No. of carboxyl groups
Coproporphyrin I	250–252	621·1	0·5	4
Coproporphyrin III?	140–144	—		

Table 4. *Properties of faecal porphyrins*

Fraction no.	Nature of porphyrin	Ester: Amount (mg.)	Ester: No. of times crystallized	Ester: M.p. (°)	Proportion of $CHCl_3$ to light petroleum in eluent from Al_2O_3	Absorption band I ($\lambda_{max.}$; mμ.)	Results from partition chromatogram: R_F value	Results from partition chromatogram: No. of carboxyl groups
F_5	Uroporphyrin	4·2	2	272–275	1:1	625·5	0·07 +trace 0·16	8 7
F_4	?	0·4	4	227–232	1:4	622·2	0·41 +trace 0·5	5 4
F_3	Coproporphyrin I Coproporphyrin III	19·5 0·8	5 3	248–252 149/168–171	1:4·5–1:5	621·1	0·5	4
F_2	Deuteroporphyrin	3·1	4	223	1:7–1:5	623·1	0·79	2
F_1	Mesoporphyrin (from protoporphyrin)	5·8	4	216–218	1:5	622·5*	0·79	2

* A sample of Fischer's mesoporphyrin IX methyl ester gave band I $\lambda_{max.}$ 623·7 mμ.

Table 5. *Composite chromatograms of faecal porphyrin esters on alumina*

(Free porphyrin soluble in 0·2% (w/v) HCl.)

Fraction no.	Band	Amount	Proportion of $CHCl_3$ to light petroleum in eluent	No. of carboxyl groups from partition chromatogram
	III	Trace	1:1	—
F_4	II	Small	1:4	5 + trace 4
F_3	I*b*	Much	1:4·5	4
	I*a*	Moderate	1:5	4

Table 6. *Properties of liver porphyrins*

Fraction no.	Porphyrin	Ester: Amount (mg.)	Ester: No. of times crystallized	Ester: M.p. (°)	Proportion of $CHCl_3$ to light petroleum in eluent from Al_2O_3	Absorption band I ($\lambda_{max.}$ mμ.)	Results from partition chromatogram: R_F value	Results from partition chromatogram: No. of carboxyl groups
L_4	Uroporphyrin	2·8	3	270	1:1	625·2	0·07	8
L_3	?	2·8	3	246–250	1:6	622·8	Mainly 0·49	4
							Also 0·63	3
L_2	?	—	3	—	1:5	622·1	Equal 0·5	4
							Amounts 0·64	3
L_1	Mesoporphyrin (from protoporphyrin)	—	3	215–218	—	622·1*	0·8	2

* See footnote to Table 4.

Fraction F_4. This yielded 0·4 mg. ester of m.p. 227–232° after four crystallizations from $CHCl_3$-methanol and contained primarily material with five ester groups (Table 4), plus a trace with four ester groups. The crystals were very similar in appearance to those from the urine fraction U_2 (Fig. 7).

The ether-insoluble porphyrin. The primary chromatogram of this material yielded mainly two bands. The first was eluted with $CHCl_3$-light petroleum ether 1:1, and the second with $CHCl_3$-acetic acid 5:1. The latter after re-esterification rechromatography and elution with $CHCl_3$-light petroleum 1:1 was combined with the former (F_5). The primary chromatogram also yielded traces of ether-soluble porphyrins which had escaped complete extraction.

Fraction F_5. This, after two crystallizations from $CHCl_3$-methanol, yielded 4·2 mg. of ester of m.p. 272–275°. The free porphyrin obtained by hydrolysis with 10% (w/v) HCl was extracted into ethyl acetate from an aqueous solution of pH 3·4. Partition chromatography showed it to consist almost entirely of material containing eight carboxyl groups and traces of porphyrin with seven carboxyl groups. Its behaviour suggests a 'uroporphyrin III'.

The fatty residue from which the porphyrins had been extracted with 10% (w/v) HCl was exhaustively examined for the presence of further porphyrins by solution in ether and extraction with 10% (w/v) NH_3. The ammoniacal extract was adjusted to pH 3·4 and treated with talc in the manner described above. On chromatography on alumina, traces of coproporphyrin and uroporphyrin esters were obtained, and a quantity of chlorophyll degradation products was eluted with $CHCl_3$. This fraction has not been further studied. No other porphyrin esters were found.

Liver porphyrins (case 2)

Ether-soluble porphyrin. The extraction of ether-soluble porphyrins proved to be only partially complete. The porphyrin soluble in 0·2% (w/v) HCl and not extracted from this solvent with $CHCl_3$ was esterified and combined with a small quantity of similar porphyrin from liver tissue (Fig. 3, Table 6, fraction L_3). A small amount of porphyrin soluble in $CHCl_3$ from 0·2% (w/v) HCl was esterified and chromatographed on alumina from which it was eluted with $CHCl_3$-light petroleum 1:5. This constituted fraction L_2, and as shown by partition chromatography contained equal amounts of porphyrins with three and four ester groups. It melted over a wide range and no precise m.p. could be obtained.

Ether-insoluble porphyrin. The primary chromatogram on alumina yielded four zones: fraction *A* was eluted with $CHCl_3$-light petroleum 1:6–1:4. After hydrolysis with 5% (w/v) HCl for 24 hr. it was extracted into ether. Fraction L_1 consisted of material extractable from ether by 5% (w/v) HCl and from the latter it could be extracted into $CHCl_3$. This material was combined with the corresponding fraction from the ether-soluble porphyrins. Treatment of the ester with HI yielded a small amount of mesoporphyrin IX ester of m.p. 215–218°. Fraction L_3 consisted of material extractable by 0·2% (w/v) HCl from ether. It was not extracted by $CHCl_3$ from 0·2% (w/v) HCl. This material was combined with the corresponding fraction from ether-soluble porphyrins. It was taken into ether, the ether removed and the residue esterified with HCl-methanol. After twice chromatographing on alumina it gave 2·8 mg. ester of m.p. 246–250° after softening at 225°. Partition chromatography showed the main fraction to contain four ester groups, but a large

Fig. 6. Coproporphyrin III methyl ester crystals from decarboxylation products (fraction U_3) ($\times 350$).

Fig. 8. Crystals of mixed coproporphyrin I methyl ester and tricarboxylic porphyrin methyl ester from liver (fraction L_3) ($\times 120$).

Fig. 5. Coproporphyrin I methyl ester crystals from decarboxylation of urinary uroporphyrin (fraction U_3) ($\times 550$).

Fig. 7. Pentacarboxylic porphyrin methyl ester crystals from urine (fraction U_3) ($\times 550$).

R. R. McSWINEY, R. E. H. NICHOLAS AND F. T. G. PRUNTY—THE PORPHYRINS OF ACUTE PORPHYRIA. THE DETECTION OF HITHERTO UNRECOGNIZED PORPHYRINS

amount of material with three ester groups was also present. The two components could not be separated on alumina columns, although there was a strong tendency for the mixture to give two bands in a similar manner to the faecal coproporphyrin fractions. The ester crystals (Fig. 8) strongly resemble those of coproporphyrin I methyl ester, except that the needles formed tend to be straighter.

Fractions *B* and *C* from the primary chromatogram were eluted respectively with $CHCl_3$-light petroleum 1:2 and 1:1 and then further fractionated on five secondary alumina chromatograms. A porphyrin ester eluted with $CHCl_3$-light petroleum 1:1 was combined with the product from fraction *D*. There were also recovered traces of porphyrin ester too small for identification. Fraction *D* formed a 'red-brown' zone at the top of the chromatogram and was eluted with $CHCl_3$-acetic acid 5:1. It was re-esterified and after two further chromatograms were run the ester eluted with $CHCl_3$-light petroleum 1:1 was combined with the product from fractions *B* and *C* mentioned above.

Fraction L_4 constituted this combined product and consisted of a total of 2·8 mg. ester of m.p. 270°. After hydrolysis with 10% (w/v) HCl it was also extracted into ethyl acetate on increasing the pH of the aqueous phase to 3·4. Partition chromatography showed that it contained only material with eight carboxyl groups. The behaviour of this porphyrin suggests that it is a 'uroporphyrin III'.

DISCUSSION

The porphyrins of the urine and the faeces of case 1 presented a similar pattern. These porphyrins were easily separable into coproporphyrin I (and a small amount of III from faeces), a pentacarboxyl porphyrin and a uroporphyrin extractable into ethyl acetate from aqueous solution of pH 3·4. Re-examination of the material obtained from a previous case (Prunty, 1946) showed that in each instance uroporphyrin from liver, faeces and urine had a similar solubility in ethyl acetate. The uroporphyrin from the liver of case 2 also shows this behaviour. Decarboxylation of the uroporphyrin obtained from the urine of case 1 showed clearly that it contained in the main uroporphyrin I, but the data from both differential crystallization and the quantitative estimation of the isomers present, suggest the decarboxylation products also contained a small proportion of coproporphyrin III. This would indicate the presence of a small amount of type III porphyrin in the original uroporphyrin ester. This ester therefore, like the ester previously obtained (Prunty, 1946), falls into the category of a Waldenström type *B* porphyrin as defined by Watson *et al.* (1945). The esters from the urine and faeces of case 1 and liver of case 2 are similar, their melting points ranging from 270 to 275°. This is rather high for so-called 'uroporphyrin III', but a figure of 275° has been reported by Watson *et al.* (1945).

The quantity of pentacarboxyl porphyrin obtained from the urine and faeces of case 1 was unfortunately insufficient for elementary analysis. It would appear to correspond with a porphyrin with a band I maximum of 622·7 mμ. mentioned by Grinstein, Schwartz & Watson (1945). The crude porphyrin ester mixture from urine also contained traces of porphyrins with six and seven carboxyl groups, but these were present in quantities too small for isolation. There was, however, neither in urine nor faeces any suggestion of the presence of a tricarboxylic porphyrin present in the liver of case 2. On the other hand, the liver did not contain detectable amounts of the porphyrins with five, six and seven carboxyl groups. Separation of the tricarboxylic porphyrin from coproporphyrin could not be achieved except by paper chromatography. There is a striking resemblance between these ester crystals (Fig. 8) and those of coproporhyrin I methyl ester illustrated by Rimington (1936).

The mesoporphyrin obtained from the liver may be derived in part from the protoporphyrin present in the blood contained in the organ. Uroporphyrin has previously been isolated from the liver by Grinstein *et al.* (1945) and Prunty (1946), and the latter author has correlated this with the presence of porphyrin granules in the liver cells as shown by ultraviolet microscopy. It must be acknowledged that the possibility remains that the coproporphyrin and tricarboxylic porphyrin were present in the blood in the organ. Notwithstanding these questions the presence of porphyrins containing 2, 3, 4, 5, 6, 7 and 8 carboxyl groups has been demonstrated, although the evidence for the presence of the members with 3, 5, 6 and 7 carboxyl groups rests for the present on their behaviour on paper partition chromatograms. Of these porphyrins those with 8, 4, 5 and 3 carboxyl groups were present in that order of decreasing quantities. It may be that these porphyrins represent intermediate steps in ascending carboxylation of the porphyrin system.

The peculiar tendency of the faecal coproporphyrin esters to separate into two bands on alumina chromatograms is worthy of note. In general increase in the proportion of chloroform in the eluent of the chromatograms of the methyl esters of these porphyrins results in an increase of the number of ester radicles in the porphyrin contained in the eluted fraction (see Tables 2, 4 and 6). The separation of the coproporphyrin esters into two bands might be due to the formation of a molecular mixture of coproporphyrin I and III esters giving rise to one of the observed bands, whereas the remainder or excess of the coproporphyrin I ester appeared as a second band below this. Watson *et al.* (1945) demonstrated experimentally that molecular combinations of esters could occur, and that the ratio of the components tended to be strictly limited. Possibly, also, similar mixtures are formed between coproporphyrin ester and tricarboxylic porphryrin ester.

It should be noted that in case 1, although during the acute attack there was an excessive excretion of

urobilinogen (Fig. 4) and both urine and faecal porphyrin excretions were high, on clinical improvement the fall in the excretion of urine and faecal porphyrin took place simultaneously. This sequence is similar to that reported before (Prunty, 1946) but is in sharp contrast to the findings of Gray, Rimington & Thomson (1948) in an unusual case of porphyria. In their case the faecal porphyrin excretion rose as the urine excretion fell, suggesting that the liver had temporarily been unable to excrete porphyrin via the biliary tract.

SUMMARY

1. The porphyrins of two cases of acute porphyria have been studied. Both uroporphyrin and coproporphyrin excretion in urine and faeces reached high values during an acute attack in the first of these cases. With clinical improvement there was a concurrent fall in these amounts and a decrease of porphobilinogen excretion in the urine.

2. Fractionation of the porphyrins present in the urine of the first case showed the presence of coproporphyrin I, a pentacarboxylic porphyrin and traces of porphyrins with six and seven carboxyl groups. The uroporphyrin was similar to 'uroporphyrin III' of Waldenström, but proved to consist mainly of uroporphyrin I.

3. The porphyrins of the faeces in case 1, in addition to protoporphyrin IX and deuteroporphyrin IX, consisted of coproporphyrin I and a small amount of the type III isomer, a pentacarboxylic porphyrin similar to that in the urine, and traces of a porphyrin with 7 carboxyl groups. There was also isolated a uroporphyrin of similar properties to that in the urine.

4. The liver of the second case contained protoporphyrin IX, and fractions consisting of mixtures of coproporphyrin and a tricarboxylic porphyrin were obtained. A uroporphyrin with similar properties to that from the urine and faeces of the first case was isolated.

5. The importance of these findings in relation to porphyrin metabolism has been indicated.

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A Note on the Urinary Uroporphyrin in Acute Porphyria

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It is well known that the urine in acute porphyria contains a substance porphobilinogen which, on standing or heating with weak acid, is converted into a brown pigment, porphobilin. There is evidence (Waldenström & Vahlquist, 1939; Prunty, 1945) that porphobilinogen may be a precursor of porphyrin also. Accordingly, methods of pretreatment of the urine in this disease to bring about maximal formation of porphyrin have been recommended by various workers: e.g. Rimington (1943*b*) states that the same full porphyrin production may be obtained by heating the weakly acidic urine on the water bath or by standing overnight with 1% (w/v) hydrochloric acid. It is, however, by no means clear to what extent such methods of pretreatment have been adopted by workers in this field or, in general, if the urine has been used fresh or after spontaneous darkening has been allowed to take place. If it could be shown that the porphyrin obtained after these treatments was identical with that present in the fresh urine, it would clearly be advantageous to apply such methods before beginning the isolation.

We are not aware, however, of any direct comparison of the porphyrins present in the fresh urine and those formed on heating.

During the course of a recent investigation of the urine from a case of acute porphyria (Gibson, Harrison & Montgomery, 1950), it was observed, as expected, that the yield of porphyrin was greatly increased by preliminary heating of the urine under weakly acidic conditions. A specimen of the uroporphyrin ester obtained after heat treatment, however, had a lower melting point than that isolated from the fresh urine, suggesting that the porphyrins might not be identical. We therefore decided to investigate further samples of urine from this patient, applying the two types of treatment mentioned above and comparing the products with the pigment preformed in the urine.

EXPERIMENTAL

Methods. Melting points were determined with a hot-stage polarizing microscope calibrated with reference substances. All melting points given in this paper are corrected. Absorption spectra were measured in a Hartridge reversion spectroscope (Beck) calibrated with a mercury arc. All solvents were dried and redistilled. Talc used for adsorption was 'acid-washed' (British Drug Houses Ltd.). Paper partition chromatography was carried out according to Nicholas & Rimington (1949), using 2:4- and 2:5-lutidine mixture and NH_3.

RESULTS

Urine samples were collected daily and kept in the refrigerator. When 6 l. had accumulated, the samples were pooled, brought to pH 3·7 with glacial acetic acid and shaken with 120 g. talc (see Grinstein, Schwartz & Watson, 1945). The talc was filtered off and the filtrate treated with 60 g. talc. The total talc was washed with acetate buffer (pH 3·7) and the pigments eluted with N-NH_3 (150, 150, 30 ml.). The combined eluates were brought to pH 3·7 with conc. HCl. In order to show that all the preformed porphyrin had been removed from the urine, a further treatment with talc was carried out. The final yield of porphyrin ester from this control was only 2 μg./l. urine.

The filtrate from the talc was divided into two parts, one half at pH 3·7 being heated to 100° and boiled for 45 min. Conc. HCl was added to the other half to give a final concentration of 1% (w/v) HCl; the mixture was allowed to stand overnight at room temperature, and then brought to pH 3·7 with NaOH. The talc treatment and elution was repeated on each half as for the fresh urine, using half quantities for each portion. The eluates from all three fractions were each extracted twice at pH 3·7 with 2 vol. 9:1 ether-light petroleum mixture (Rimington, 1943*a*) to remove any coproporphyrin. Repeated extraction of the ether layers with 0·25% (w/v) HCl did not indicate the presence of any significant amount of coproporphyrin as judged by colour and fluorescence of the extracts in ultraviolet light.

The ether was allowed to evaporate from the aqueous phase of the urine filtrates, the porphyrin adsorbed from each fraction on successive small quantities of talc (total 2·5 g. talc/l. urine), and eluted with several small portions of N-NH_3 (total vol. 10 ml./l. urine). The eluates were brought to pH 3·7 with conc. HCl and allowed to stand overnight. The precipitates were centrifuged off and the porphyrin in each esterified by standing overnight with 5 ml. methanol freshly saturated with HCl. The remaining insoluble residues were centrifuged off and retreated with fresh methanolic HCl. This treatment was repeated a third time. The combined acidic methanol fractions were mixed with an equal volume of $CHCl_3$, shaken with an excess of ice water and centrifuged. The $CHCl_3$ was washed three times with water, once with dilute NH_3 and twice more with water. It was dried with anhydrous Na_2SO_4, filtered and taken to dryness on the water bath. The residues were dissolved in a few drops of $CHCl_3$, mixed with a few ml. benzene and chromatographed on chalk columns, development being carried out with $CHCl_3$-benzene (1:20). The brown pigment forming the upper zone on each column was discarded and the porphyrins eluted with $CHCl_3$. The latter was evaporated, the residues dissolved in benzene and rechromatographed on chalk.

The findings for the three fractions were as follows.

Fresh urine. On chalk a single porphyrin band was observed. It was eluted with chloroform and yielded 3·68 mg. ester from 6 l. urine, crystallizing from chloroform-methanol (1:3) as long matted hairs. After three further crystallizations these melted at 282–285°. In chloroform the absorption bands were centred at 625·5, 569·4 (max.), 535·1, and 501 mμ. Paper chromatography of the free porphyrin made by saponification with hot 10% (w/v) sodium hydroxide gave a single spot corresponding in position with a uroporphyrin. Decarboxylation was carried out by heating to 185° in 1% (w/v) hydrochloric acid for 3 hr. The product was esterified with methanolic HCl, chromatographed from benzene-light petroleum (1:1) and developed with chloroform-benzene (1:20). The chromatogram showed one main band, which gave compact clumps of needles from methanol-chloroform (5:1), melting after two recrystallizations at 248–250°. In chloroform bands were present at 621·5, 596·2, 566·6 (max.), 534·1 and 498·5 mμ. The free porphyrin gave one spot on paper chromatography, corresponding in position with a coproporphyrin. The material appears to be mainly coproporphyrin I, and this, coupled with the findings above, indicates that the main porphyrin originally present in the urine was uroporphyrin I. We did not find evidence for the presence of a heptacarboxylic porphyrin in the urine.

Boiled urine. On chromatography, three main bands were observed, one large red band and, further down the column, two smaller violet bands. The total weight of methyl ester from 3 l. urine was 12·46 mg., equivalent to seven times as much as from the fresh urine. (The increased yield on heating has been confirmed several times.) The main band was separated from the two smaller bands, and gave

short hair-like crystals melting after three recrystallizations from chloroform-methanol (1:3) at 264–265°. The ester in chloroform gave bands at 625·6, 565·6 (max.), 533·8, and 499 mμ. Paper chromatography of the free porphyrin gave two spots, one corresponding with uroporphyrin, and a weaker one with a slightly greater R_F value. After decarboxylation and esterification, three bands were obtained on a chalk chromatogram, one at the top of the column, then a narrow band, and finally a broad main band. The main band yielded needles melting after three crystallizations at 235–243°. The bands in chloroform were at 621·9, 595·9, 566·7 (max.), 533·2, and 499 mμ. The free porphyrin on paper gave two spots very close together and corresponding with coproporphyrins. Judging from the melting point the material contains much coproporphyrin I, but accompanied by some impurity. The results suggest that the porphyrin of this fraction contained uroporphyrin I mixed with some other porphyrin not separable on chalk. It appears to correspond closely with Waldenström Type *B* porphyrin (Watson, Schwartz & Hawkinson, 1945). The two minor violet bands from the uroporphyrin chromatogram, together containing less than 0·5 mg. ester, gave absorption bands in chloroform at 624·9, 569·8 (max.), 536·1, and 500 mμ. The material was freely soluble in hot methanol and deposited short needles on cooling. After four recrystallizations from methanol it melted at 233–235°, the final crystals being rosettes of small bent needles. Paper chromatography of the free porphyrin obtained by hydrolysis of the initial eluate of these minor bands with concentrated hydrochloric acid gave three rather indefinite spots, the main one of which appeared to correspond in position with a pentacarboxylic porphyrin and which may be similar to the porphyrin, m.p. 227–232°, described by McSwiney, Nicholas & Prunty (1949). The amount of material available was not sufficient for further characterization.

Hydrochloric acid-treated urine. This fraction gave a yield of only 75 μg. porphyrin from 3 l. urine. It gave a typical uroporphyrin spectrum, but was not further identified.

DISCUSSION

Our results suggest that the porphyrin obtained after boiling is not a single substance, and that the main fraction is not the same as that obtained from the fresh urine. It seems possible that the changes taking place on allowing porphyria urine to stand and darken in colour might similarly be accompanied by formation of porphyrin different from that present when it is fresh. The work confirms our previous finding that the main uroporphyrin in the urine of this patient is of type I and not of type III. The latter was generally considered to be characteristic of acute porphyria until the recent work of Watson *et al.* (1945). It may be noted that the main porphyrin ester from the boiled urine gave a melting point close to that usually ascribed to the Waldenström porphyrin ester. The result obtained on decarboxylation of this porphyrin did not, however, suggest that it contained any large proportion of uroporphyrin III, though the main product separated after decarboxylation was not pure coproporphyrin I. The melting point of the latter was lower than that obtained for the coproporphyrin derived from the fresh urine fraction, and it melted over a range rather than sharply.

It is perhaps of interest to record that we did not detect in this case the heptacarboxylic porphyrin (m.p. 208°) reported by Grinstein *et al.* (1945) in some samples of acute porphyria urine. It is possible that a trace of heptacarboxylic porphyrin was present in the boiled urine as shown by the weak spot given on paper chromatography with an R_F value slightly greater than that of uroporphyrin.

In general, our results point to the conclusion that while preliminary boiling of the weakly acid urine greatly increases the yield of the uroporphyrin fraction, this treatment has the drawback that it may give a false picture of the nature of the uroporphyrin originally present in the fresh urine. It is obviously impossible to draw any general conclusions from the study of a single patient but, in view of the rarity of porphyria, it seems desirable to record our observations in the hope that they may be supplemented by workers having access to a wider range of material.

SUMMARY

1. Uroporphyrin was isolated from the fresh urine of a case of acute porphyria and shown to be type I uroporphyrin. No other uroporphyrins were found.

2. Preliminary treatment of the urine by boiling in weakly acidic solution, until maximum development of colour, led to a seven-fold increase in the uroporphyrin fraction, but this newly formed fraction consisted of several different uroporphyrins and pure uroporphyrin I could not be isolated from it.

We wish to thank Dr D. A. D. Montgomery for obtaining the urine samples for us.

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The Site of Action of Penicillin

1. UPTAKE OF PENICILLIN ON BACTERIA

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Since the discovery of penicillin considerable data have been published about its mode of action. Among the findings which have a real bearing on the primary action of the drug on bacterial metabolism is the early observation that penicillin does not affect resting cells, but gradually inhibits the respiration of rapidly growing cells (Chain & Duthie, 1945). This would indicate that the killing effect is associated with the act of division, and for this reason studies with isolated enzyme systems are unlikely to progress far unless the enzymes studied are closely connected with cell division.

Gale & Rodwell (1949) have shown that by training a sensitive organism to dispense with certain amino-acids it becomes more resistant to penicillin without any contact with the drug. It appears that if an organism requires energy to transport preformed amino-acids from the external environment it will be sensitive to penicillin. In the case of one such amino-acid (glutamic acid, Gale & Taylor, 1947) it was found that penicillin prevented active transport across the cell wall. Active growth of the organism for several hours in the presence of penicillin is required for the prevention of amino-acid transport. This prevention may, therefore, be an end result of a series of reactions instituted by the penicillin, and is very probably still a secondary effect of the drug, although it may occur earlier than many other effects noticed. It remains to be seen whether this phenomenon is actually the cause of death of the organism.

The present studies aimed at examining the primary action of penicillin on the bacterial cell. The penicillin molecules which affect bacteria may become attached to them. Hobby, Meyer & Chaffee (1942) were unable to show any definite removal of the drug by bacteria when using biological assay methods, and so a more sensitive method would be required to detect the minute amounts of penicillin which may be involved. The incorporation of a radioactive element in the antibiotic seemed a likely method of affording a high sensitivity, and had the added advantage that the fate of the penicillin could be followed after reaction with the organism. Radioactive ^{35}S was added to a synthetic penicillin medium, and the crude labelled penicillin extracted from the culture filtrate was utilized in our experiments. Initially some difficulties were encountered owing to the very small amount of penicillin removed by the bacteria, and to the very soft radiations of ^{35}S (Rowley, Miller, Rowlands & Lester Smith, 1948). However, by using penicillin of the highest specific activity and by directly measuring the radioactivity on thin layers of dried bacteria, consistent results have been obtained (Cooper & Rowley 1949).

EXPERIMENTAL

Preparation of radioactive penicillin

The chemically defined medium of Jarvis & Johnson (1947) for the growth of the mould has been successfully modified by decreasing the S content to about a quarter, thereby deliberately sacrificing the total yield of penicillin in favour of a higher yield calculated on the S. The radioactive S in most instances was supplied as Na_2S, which was converted to sulphate by boiling with ammonia and redistilled H_2O_2. After inoculation with a loopful of vegetative mould suspension, the medium was shaken for about 4 days at 24° and

filtered free from mycelium. The broth and washings were cooled in ice, acidified to pH 2 with HCl, and rapidly extracted several times with ice-cold ether. The penicillin was extracted from this with dilute phosphate buffer at pH 7·5 containing 0·1% of sodium hexametaphosphate to stabilize the penicillin.

Table 1 shows the quantities used and yields obtained. The penicillin was dispensed in ampoules (40 units), freeze dried and stored in the refrigerator. The biological and radioactivities were redetermined at weekly intervals.

Table 1. *Yields of radio penicillin obtained in five fermentations*

Vol. medium (ml.)	^{35}S concn. (μc./ml.)	Penicillin yield (units)	Conversion of ^{35}S (%)	Specific activity (μc./unit)	Specific activity (Counts/unit)
2	50	330	20	0·060	12,100
10	50	2500	18	0·037	7,500
10	88	1500	16	0·095	19,000
2	250	220	8·0	0·183	37,000
5	100	420	5·3	0·063	12,800

Uptake of penicillin by bacteria

At least 50 mg. of dry bacteria were required for each experiment, and the organisms were harvested from 16 hr. cultures on agar plates and employed in concentrations of 0·1–10 mg. dry wt./ml. of medium. None of the organisms employed produced penicillinase.

To measure uptake, a bacterial suspension in broth was divided into several equal portions (generally 2 ml.) and incubated for 1 hr. Radio penicillin was added in varying amounts, and the suspensions were incubated for a further hour. The organisms were then centrifuged at about 1500 rev./min. for 20 min., washed once and resuspended in distilled water.

Samples of these suspensions (0·1 ml.) were immediately transferred to 9·9 ml. of formol saline for turbidimetric assay on a Hilger absorptiometer, calibrated in terms of bacterial dry wt./ml. Similar portions placed on 1 cm. nickel planchettes were dried at 120° for 20 min. and kept in a desiccator until counted. Spreading of the samples was readily achieved by diluting with distilled water until the suspension covered all the surface of the planchette, when the bacteria settled evenly and were not disturbed by drying. Under these conditions the sample layer was never thicker than 0·3–0·6 mg./sq.cm., when self-absorption effects were found to be negligible.

When they had been sampled for turbidimetric and total radioactive assay, the bacterial suspensions in distilled water were centrifuged and the clear supernatant was counted. This usually contained negligible radioactivity. The difference between this count and that of the suspension as a whole was taken to be the radioactivity attached to the bacteria. This difference method would tend to reduce possible errors from background variation, and is equivalent to counting background for a time equal to that taken over each bacterial suspension.

The activity was low, about 10 counts/min. above background, which was reasonably constant at 14 counts/min. Either nine or five samples of each suspension were counted for 5 or 10 min. respectively, giving a total of about 500 counts above background from which the probable statistical error to be expected is $\pm 7\%$. This, with an error of $\pm 3\%$ from the absorptiometer readings and an unknown error in taking samples, produced results which varied within the approximate limits of $\pm 20\%$.

Uptake has been expressed as International penicillin units/g. dry wt. of bacteria.

Assay of radio penicillin. The radioactive assays were performed with standard equipment and using well-known techniques. The equipment consists of a thin mica window Geiger Müller counter (approximately 2 mg./sq.cm.; General Electric Co. Ltd) operating into a T.R.E. type 1014 probe unit, the counts being registered by a type 200A. scaler. The resolving time of the probe unit was set at 400 μsec., the counting rate being sufficiently low (below 600 counts/min.) to neglect any correction from this source of error.

Total counts were of the order of twice the background. Although the use of 'infinitely thick' layers of material on the planchette would have increased the counting rate, the measurements were confined to thicknesses below that at which self-absorption corrections became necessary. This choice was mainly dictated by the small quantity of radio penicillin available, together with the difficulty of placing sufficient material on the planchette. An experimental curve was derived relating loss of counts from self-absorption with wt./unit area of material on the planchette. It was found that a thickness of 1·0 mg./sq.cm. gave an error of approximately 5%. The layer thickness used in the experiments never exceeded 0·6 mg./sq.cm. and were generally much less, thus giving a negligible correction.

All counts were comparative, and the radioactivity of the penicillin was assayed at weekly intervals. A ^{35}S standard, giving approximately 500 counts/min., was counted at regular intervals together with a background count, enabling a decay curve of ^{35}S to be plotted. This with a U standard count provided a rough check on the operation of the equipment. The geometrical arrangement below the window of the counter was identical in all the experiments, thus ensuring constant efficiency and scatter. The planchettes employed were sufficiently thick for the back scatter to be saturated.

RESULTS

Variation in uptake with concentration of penicillin in medium

Bacteria are unable to divide when incubated in broth above a certain bacterial concentration (the '*M* concentration', Bail, 1929). Under these 'resting' conditions (e.g. 3 mg. dry wt./ml.) and in saline, the penicillin taken up on the bacteria was found to increase with the concentration of penicillin both with sensitive and with resistant organisms. Four sensitive staphylococci tested differed in shape of uptake curve from two resistant staphylococci, and

from *Escherichia coli* and *Klebsiella pneumoniae*, at penicillin concentrations less than 0·1 unit/ml. (Fig. 1 shows two typical curves).

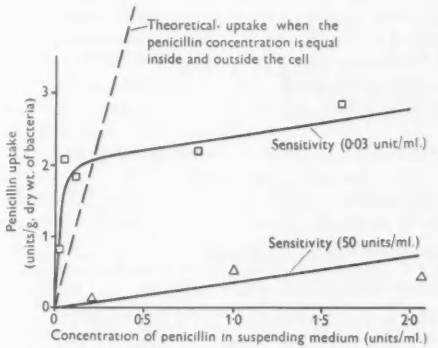
Theoretical uptake when the penicillin concentration is equal inside and outside the cell
Sensitivity (0·03 unit/ml.)
Sensitivity (50 units/ml.)
Penicillin uptake (units/g. dry wt. of bacteria)
Concentration of penicillin in suspending medium (units/ml.)

Fig. 1. Penicillin uptake on resting sensitive (–□–□–) and resistant (–△–△–) staphylococci on suspension in solutions of the drug. The theoretical uptake is calculated on the assumption that all the water removed by drying the cells at 120° is available for solution of the penicillin.

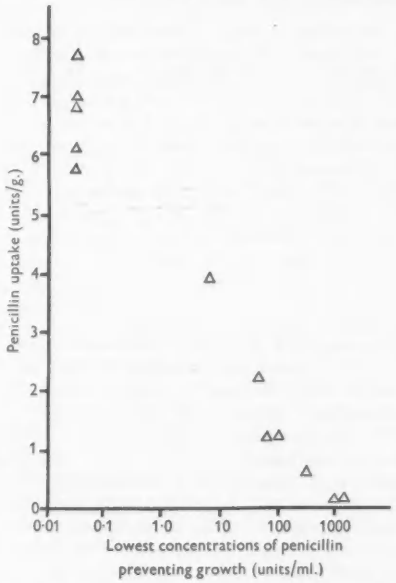

Fig. 2. Relation between penicillin uptake and penicillin sensitivity of rapidly growing bacteria.

The penicillin concentration attained inside the cell at these low titres was much greater than that in the external environment with sensitive organisms, but much less in the case of resistant ones.

Under conditions of rapid division the uptake was greater, and even quite resistant organisms could concentrate penicillin from the medium to some extent. When 0·04 unit/ml. of radioactive penicillin was incubated for 1 hr. with a rapidly growing culture, the uptake was found to increase with the sensitivity of the strain (Fig. 2).

Uptake with time

If radio penicillin is added to a 'resting' culture of a sensitive staphylococcus, which is then immediately centrifuged, the uptake is found to be essentially the same after 3 min. as after longer contact times.

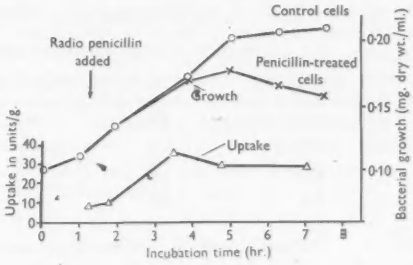

Fig. 3. Rate of penicillin uptake (–△–△–) on rapidly growing sensitive staphylococci, compared with rate of growth in presence (– × – × –) and absence (–○–○–) of the drug. At the point ↓ radio penicillin was added up to a final concentration of 0·04 unit/ml.

If, on the other hand, the cells are rapidly dividing the initial uptake is greater, and then increases at a measurable rate to a value over tenfold that of the 'resting value' (Fig. 3), reaching a maximum shortly before growth is stopped. Thus it can be seen that a rapid initial uptake occurs under all conditions of contact, and this uptake is augmented when the cell is allowed to divide in the presence of penicillin. The initial rapid uptake is as complete at 0° as at 37°, within the 3 min. required to carry out a determination.

Attempts to prevent penicillin uptake

Penicillin-sensitive staphylococci in saline were treated with various agents for 30 min. at 37°, washed twice with saline and resuspended in broth at a concentration of 3 mg. of bacterial dry wt./ml. After 1 hr. incubation, radioactive penicillin was added to a concentration of 0·1 unit/ml. and after a further hour at 37° the organisms were centrifuged and washed prior to counting.

Results presented in Table 2 show that mere death of the cell was not sufficient to prevent its 'resting uptake', neither was the centre reacting with penicillin blocked by pretreatment with formaldehyde, CTAB, euflavin or hydrochloric acid.

Table 2. *Effect of some agents on the ability of sensitive staphylococci to take up penicillin from a 0·1 unit/ml. solution*

(Bacteria were allowed 0·5 hr. contact with agent and washed twice before the uptake was determined.)

Agent	Concn. (%, w/v)	Uptake on control (units/g.)	Uptake after treatment (units/g.)	Viability of cells after treatment with agent
Formalin	3 (v/v)	3·8	3·7	–
CTAB	0·05	4·4	2·3	–
Crystalline penicillin	200 unit/ml.	4·4	0	+
Euflavin	0·03	4·4	3·1	+
Acetic anhydride	10	11·5	0·5	Some survivors
Buffered acetic anhydride	10	11·5	0·4	+
Buffered acetyl chloride	10	11·5	1·8	+
Hydrochloric acid	N	11·5	9·1	Some survivors

Table 3. *Non-removal of penicillin from sensitive staphylococci by repeated washing in distilled water*

(Suspensions were allowed to stand for 20 min. between each centrifuging.)

Penicillin concn. from which uptake took place (unit/ml.)	Penicillin remaining on bacteria after 4 washings (units/g.)				Mean uptake (unit/g.) (average of columns 2–5)
	1st	2nd	3rd	4th	
0·25	1·8	2·2	2·4	2·9	2·3
1·5	3·2	2·9	3·1	3·9	3·3
3·0	2·7	4·5	3·3	3·4	3·5
7·2	4·0	5·5	6·2	4·8	5·1

Table 4. *Inability of several agents to remove penicillin from sensitive staphylococci*

Agent	Concn. (%, w/v)	Living cells after 1 hr. contact with penicillin (units/g.)		HCHO-treated cells after 1 hr. contact with penicillin (units/g.)		Living cells after 5 min. contact with penicillin (units/g.)		Cells rapidly grown with penicillin for 1 hr. (units/g.)	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated
Aerosol OT	0·1	3·0	3·0	3·4	1·7	4·3	3·1	5·2	6·6
CTAB	0·1	3·0	2·6	3·4	5·3	4·3	2·5	—	—
Penicillin	0·1	3·0	1·3	3·4	4·6	4·3	3·2	5·2	4·7
Sodium taurocholate	0·7	3·0	2·7	3·4	2·2	4·3	3·7	—	—
Cysteine HCl	1	—	—	—	—	4·3	4·2	—	—
Glucose	1	3·0	5·4	—	—	—	—	—	—
Phenol	5	4·5	3·2	—	—	—	—	—	—
NaOH	N	3·0	3·1	—	—	—	—	—	—
HCl	N	3·0	3·4	—	—	—	—	—	—

However, pretreatment with ordinary penicillin, or buffered acetic anhydride, completely prevented uptake within experimental error.

In an attempt to remove the 'active centre' from the cells along with the Gram-positive complex (Henry & Stacey, 1948), it was found that sensitive staphylococci which had been heated at 60° for 5 days in saline alone as well as with 1 % taurocholate, were unable to take up penicillin in 0·1 unit/ml.

Attempts to remove attached penicillin from staphylococci

Sensitive staphylococci (3 mg. dry wt./ml. in broth) were incubated with various concentrations of radio penicillin for 1 hr., centrifuged and washed. After radioactive and turbidimetric assay, the suspension was rewashed with distilled water and recounted four times. No change in attached radioactivity was found (Table 3). Washing was similarly ineffective in removing radioactivity from resistant bacteria. Other samples of sensitive staphylococci were similarly treated with radioactive penicillin at 0·1 unit/ml., washed and incubated for 30 min. in saline with various agents to see if they removed the radioactivity. Samples were assayed after a further washing. None of the agents tested were effective in removing radioactivity from living or formaldehyde-killed staphylococci after 1 hr. contact or from cells rapidly grown for 1 hr. in presence of penicillin (Table 4).

However, it was found that within experimental error the bacteria completely lost any attached radioactivity after heating for several days in saline at 60°; furthermore, the organisms were unable to reabsorb any more penicillin from a 0·1 unit/ml. solution after this treatment.

DISCUSSION

All bacteria tested could take up penicillin to some extent. Resting, penicillin-sensitive bacteria could concentrate the drug from the external into the internal environment only at low external penicillin concentrations. With resting sensitive organisms at higher concentrations and with resistant organisms at all concentrations, the penicillin was always at a lower level inside than outside the cells.

This ability of sensitive bacteria to concentrate the drug from the medium cannot be explained as a simple diffusion across the cell wall. Furthermore, this high uptake is not a general property of all bacteria, so that it must be assumed that sensitive organisms possess a special centre which has a fairly high affinity for the drug.

That this concentration of radioactivity into the cell is directly related to the antibacterial action of penicillin is borne out by the following points. (1) It occurs only from 0–0·1 unit/ml., i.e. over the minimal concentration range in which penicillin-sensitive organisms are killed; the centre is apparently saturated at higher concentrations. (2) With a fixed concentration of penicillin in the medium, the uptake increases with the sensitivity of the strain. (3) Pre-treatment with ordinary crystalline penicillin prevents attachment of radioactivity from low concentrations, while CTAB and other compounds do not. This uptake is not, therefore, a general surface property, but is specific to the penicillin molecule. (4) Much more radioactivity becomes attached when the organisms are grown in presence of the drug. It is known that active growth in presence of penicillin is necessary for it to exert its antibacterial action.

The radioactivity is not removed by repeated washings, by detergents or by ordinary crystalline penicillin. As penicillin itself is known to possess only feeble surface activity, any adsorbed penicillin should be removed by these treatments. It therefore seems unlikely that the reaction of penicillin with the cell is anything but a direct chemical combination with a cellular component; it does not seem to be an equilibrium reaction. This component is present in minute amounts in resting cells, but more appears to be produced during growth.

Some attempts were made to discover the chemical nature of this component by pretreatment of the cell with various reagents. Such experiments are inconclusive, as it is uncertain that the reagent will necessarily be able to affect the centre, or that any blockage noticed will not be due to a steric effect following reaction with a nearby group, but it is interesting to note that acetylating agents prevent uptake, while formaldehyde does not. Maas & Johnson (1949) have recently been able to demonstrate an uptake of firmly bound penicillin of a similar order to that reported above, using both biological and radioactive methods of assay. They were unable to correlate the uptake of the drug with sensitivity of the organism to penicillin and they say that it is entirely possible that this uptake may not be connected with the action of penicillin.

SUMMARY

1. The preparation of radioactive penicillin is described.

2. The amount of penicillin which becomes attached to bacteria under various conditions has been measured, and the uptake most probably responsible for its antibacterial activity has been distinguished.

3. This uptake appears to be due to a direct chemical reaction with a cellular component present in minute amounts in resting bacteria, more of which becomes available to the penicillin during growth.

Our thanks are due to Sir Alexander Fleming, F.R.S., for his continued encouragement with this work.

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The Effect of High- and Low-plane Feeding on the Serum Protein Levels of Pregnant Ewes, Foetuses and Young Lambs

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This communication presents some results obtained in a large-scale investigation of the effect of different dietary levels on the health and wellbeing of pregnant ewes and their lambs. Other aspects have already been described elsewhere (Thomson & Thomson, 1949). The data recorded here are the serum protein levels of the blood samples of pregnant ewes and their foetuses at approximately 10 days before term, and of lambs at varying brief intervals after birth. Certain figures obtained from blood samples of pregnant sheep and foetuses not included in the nutritional experiment are recorded with a view to supplying as much data as possible on such sera.

EXPERIMENTAL

Management of animals

The sheep used in the nutritional experiment were half-bred ewes crossed with a Suffolk tup and were about 1 yr. old at tupping. These animals were tupped while at grass and were only brought indoors for controlled feeding at about the sixth week in pregnancy. From then on they were divided into two groups, one being fed on a high plane and the other on a low plane of nutrition. Both groups received the same amounts of chop (oat straw and hay) and cod liver oil, but the diets differed both in energy and protein content due to differences in the amounts of roots and meals mixture fed. These diets are described in Table 1.

Table 1. *Daily rations of 'high'- and 'low'- plane ewes*

	High plane	Low plane
'Chop' (lb.)	0·5	0·5
Meals (lb.)	1·4 up to 2·0	0·75 down to 0·6
Roots (lb.)	4·2 up to 5·1	2·25 down to 1·8
Cod liver oil (ml.)	5	5
Gross digestible energy (lb.)	1·67 to 2·20	1·02 to 0·82
Protein equivalent (lb.)	0·19 to 0·25	0·11 to 0·08

The meals mixture consisted of ground maize, 4 parts; ground oats, 1 part; linseed cake meal, 4 parts; ground chalk, 2% (w/w); salt, 1% (w/w). The feeding was designed so that, by varying the meals intake over the pregnancy period from the sixth week to term, the high-plane ewes should gain about 40–50 lb. in weight, whilst the low-plane ewes should about maintain their weight. Actually the animals referred to in this communication put on an average of 47 lb. in the high-plane group and 7·5 lb. in the low-plane group. It should be noted that the small increase in the low-plane group and the large increase in the high-plane group is, in part, due to the fact that the ewes were immature and still growing.

Operative technique. At approximately the tenth day before the calculated date of parturition the individual ewes were submitted, under chloral hydrate anaesthesia, to Caesarean section and, whilst still alive and before the foetus was removed from the uterus, samples of foetal blood were obtained from the umbilical vein and samples of maternal blood from the jugular vein. In the case of twins, samples were obtained separately from each foetus.

Analytical methods

Total serum protein concentration. This was measured by determination of total N by a micro-Kjeldahl method, using SeO_2 as a catalyst and employing a Markham distillation apparatus.

Albumin total protein ratios. The globulins were precipitated by saturating with $MgSO_4$: to 1 ml. of serum were added 1·2 g. of finely powdered $MgSO_4$ and 7 ml. of a saturated aqueous solution of $MgSO_4$. The mixture, with occasional shaking, was left at room temperature for 24 hr., after which it was filtered through a Whatman no. 42 filter paper and the albumin N was determined on a sample of the crystal-clear filtrate.

Electrophoresis. The serum samples were dialysed against phosphate buffer, pH 8, $I = 0{\cdot}2$ at 0°. In one case a veronal buffer (Michaelis, 1930) of pH 8·6 was used. The difference in refraction between the inner fluid (n_1) and the outer (n_0) was measured with a dipping refractometer. The dialysed sera were examined in a Tiselius (1937) apparatus, optical observations being made with the diagonal schlieren method and monochromatic light (546 mμ.). The relative concentration of the components was measured by optical projection, tracing of photographs, extrapolation and planimetry. Anode and cathode limb patterns were used wherever possible, and in many cases two or more patterns from each limb obtained at different points of the run were analysed. Discrepancies in the results obtained from both limbs of the U-tube are expressed in subsequent tables as the range of error, although this may actually be greater (Svensson, 1946). In some cases where light dispersion due to lipid was marked, e.g. lamb and sheep sera, the sera were defatted by the method of McFarlane (1942). The double-length section cell described by Longsworth, Cannon & MacInnes (1940) was frequently employed as the sheep globulins showed poor separation.

RESULTS AND DISCUSSION

The quantitative findings for the two experimental groups of animals are shown in Table 2. It will be seen that the blood sera of the ewes in the high-plane

Table 2. *Protein content of sera of ewes and foetuses (10 days before parturition) in relation to plane of nutrition*

	Ewe			Foetus		
		Albumin/total protein as percentage determined by			Albumin/total protein as percentage determined by	
Ewe no.	Total protein (g./100 ml.)	$MgSO_4$ ppt.	Electrophoresis	Total protein (g./100 ml.)	$MgSO_4$ ppt.	Electrophoresis
			High-plane ewes			
11	4·93	—	—	4·30	—	—
44	4·75	74·3	—	4·01	90·5	—
76	4·98	66·2	—	F_1 4·12*	98·0	—
				F_2 3·94	98·0	—
71	4·40	68·1	—	3·36	92·4	—
36	5·63	65·0	—	4·47	78·3	—
33	6·09	61·1	—	4·39	85·4	73·0±2
39	4·94	73·5	67·3†	4·13	84·3	69·2†
Mean	**5·07**	**68·0**	—	**4·09**	**89·6**	—
S.D.	0·54	—	—	0·34	—	—
			Low-plane ewes			
43	4·75	—	—	4·24	—	—
40	4·31	65·4	—	3·70	87·6	—
23	3·96	66·1	—	F_1 3·46	88·4	—
				F_2 3·38	84·0	—
32	4·65	70·1	—	3·75	86·9	—
55	4·12	66·7	—	3·52	76·1	—
49	4·69	72·0	62·6±0	3·94	83·2	75·0±3
69	4·04	71·0	—	F_1 2·71	100·0	65·0±4
				F_2 3·01	84·7	77·0‡
Mean	**4·36**	**68·6**	—	**3·52**	**86·4**	—
S.D.	0·34	—	—	0·46	—	—
t	2·93	—	—	2·85	—	—

* F_1 and F_2 refer to twin lambs. † Analysis of cathode side only. ‡ Analysis of anode side only.

Table 3. *Lamb sera analyses in relation to plane of nutrition of their dams*

			Albumin/total protein as percentage determined by		
Ewe no.	Lamb no.	Total protein (g./100 ml.)	$MgSO_4$ ppt.	Electrophoresis	Age of lamb (days)
		Lambs from high-plane ewes			
42	110	6·62	38·7	32·5±1	1
30	93	5·86	53·2	47·3±3	4
52	100	5·92	55·2	43·9±1	4
51	97	5·16	69·8	55·5±2	10
25	106	6·04	64·6	51·8±1	10
	Mean	**5·92**	**56·2**	—	—
	S.D.	0·52	—	—	—
		Lambs from low-plane ewes			
28	102	4·12	70·9	65·0±4	1
53	105	4·74	62·0	38·4±1	1
22	82	5·96	47·0	—	4
34	87	5·10	54·9	—	4
26	88	4·24	76·4	62·6	10
37	92	4·08	—	—	10
	Mean	**4·71**	**62·2**	—	—
	S.D.	0·73	—	—	—
	t	3·10	—	—	—

group had a higher total protein content than the sera of the low-plane ewes. This difference was statistically significant, the '*t*' value being 2·93. The mean value for albumin nitrogen expressed as a percentage of total nitrogen showed no significant difference between the groups. Similarly, for the sera of the foetuses, those from the high-plane group were significantly higher in total protein than those from the low-plane group, the '*t*' value being 2·85. The slight difference between the mean albumin/total protein ratios of the two groups was not significant. The few comparisons possible between the results

obtained for the albumin/total protein ratios, as obtained by the magnesium sulphate precipitation method and by electrophoresis, show, in every case, a lower result by the second method. Such differences are known to occur on account of solubility characteristics of the proteins and they are possibly accentuated in this instance, owing to the adoption of unfavourable conditions such as degree of dilution and buffer ionic strength in the electrophoretic analyses of the sera of this particular species.

Table 3 shows the results for samples of serum obtained from lambs of the two groups at different intervals up to 10 days after birth. All the lambs had been allowed to suckle normally in the interval. Here again the mean value, 5·92 g./100 ml., for the total protein of the sera of the lambs from the high-plane ewes was significantly higher than the mean value, 4·71 g./100 ml., for the sera of the lambs from the low-plane ewes, the 't' value being 3·10. The mean values for the albumin/total protein ratio show a slight but not significant difference between the two groups. For each group they are, however, lower than the corresponding values for the sera from either ewes or foetuses from the same groups. Since,

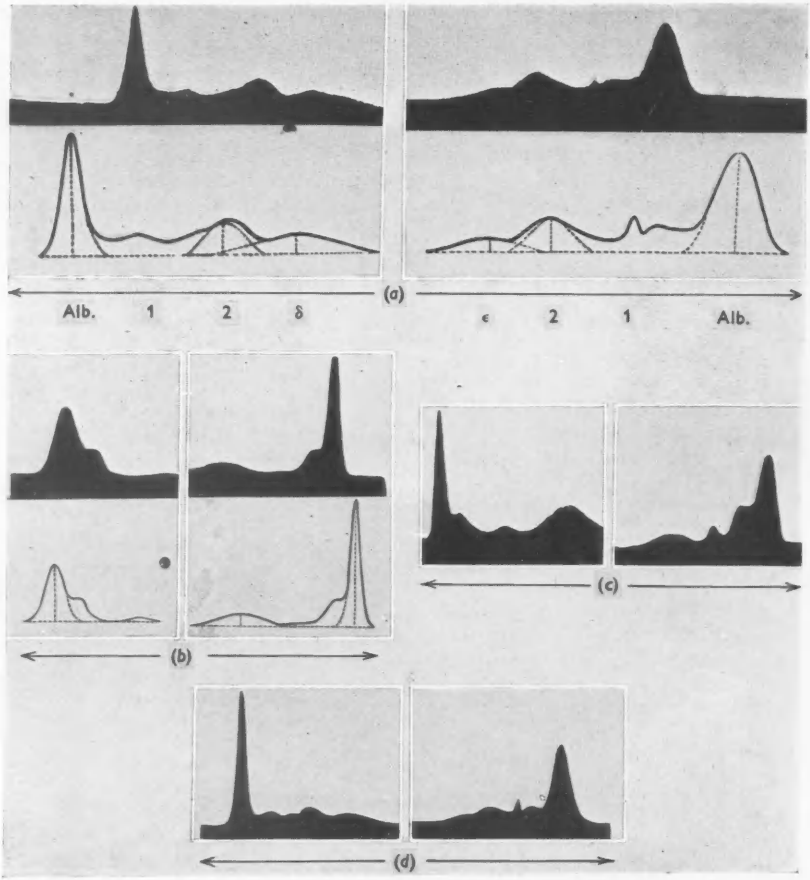

Fig. 1. Electrophoresis patterns of adult and foetal sheep sera. *a*, normal sheep serum and method of extrapolation. *b*, serum of foetus about 10 days before term from low plane-fed ewe. *c*, serum of 112-day foetus from sheep on pasture. *d*, serum of ram on pasture.

however, a globulin fraction appears in the serum of newborn lambs on suckling, as has recently been shown by Charlwood & Thomson (1948), this might be expected. These authors published electrophoretic patterns of sera from sheep and from their lambs before and after suckling, but did not give any quantitative data.

In our experiments, electrophoretic analyses of sera from sheep at different stages of pregnancy, from foetuses and from newborn lambs 1, 4 and 10 days old were made. Fig. 1*a* shows a typical electrophoretic pattern of ewe serum. As efforts to resolve all the components by extrapolation were unsuccessful the method of analysis outlined was

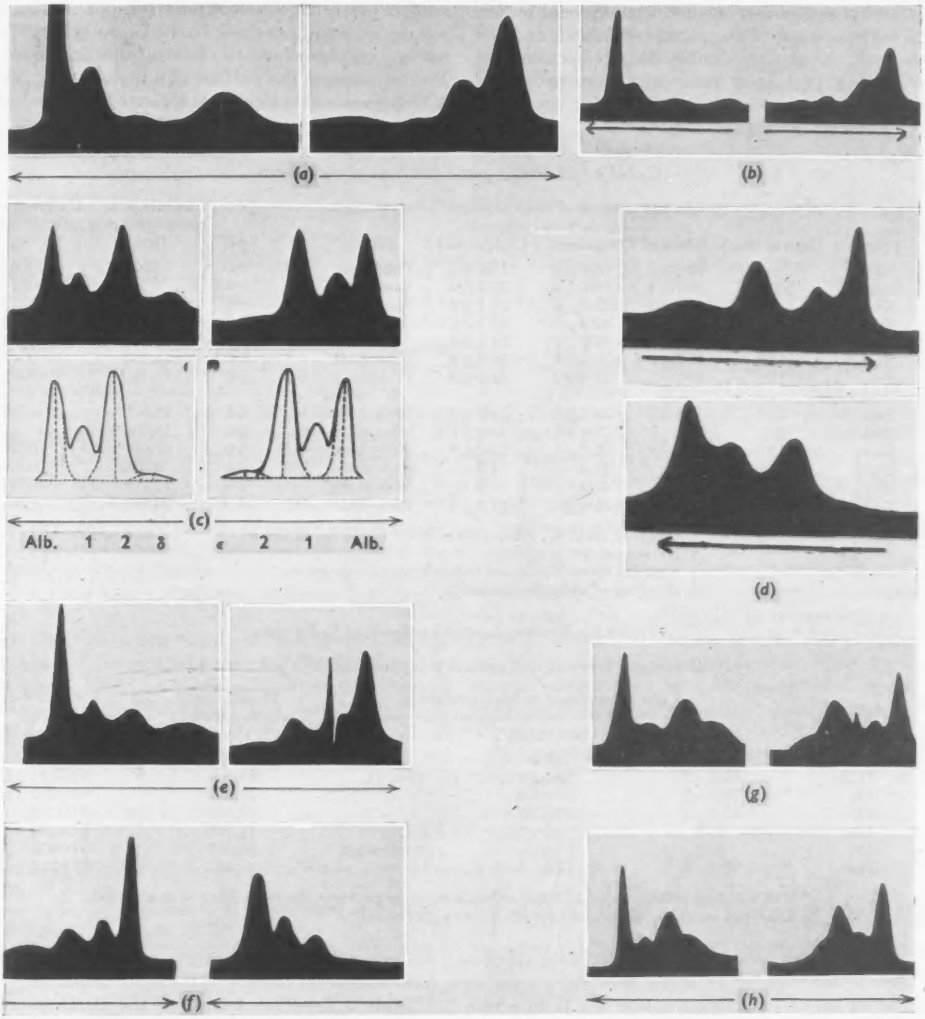

Fig. 2. Electrophoresis patterns of lamb and sheep sera. *a*, serum of unsuckled lamb of pasture-fed ewe. *b* and *c*, sera from two 1-day-old lambs from different low-plane fed mothers. The method of extrapolation is shown. *d*, serum of 4-day-old lamb from high-plane mother. *e* and *f*, serum of two 10-day-old lambs from high-plane mothers; *f*, after defatting. *g*, normal, and *h*, defatted serum of ewe at 112 days pregnancy.

used. Only the albumin and the slowest peak component 2 were measured and the intermediate part of the pattern, obviously not homogeneous, was termed for convenience component 1. Although better separation was effected with veronal buffer, the indistinctness of the picture obtained, and the introduction of the double length section cell facilitating prolonged runs with the phosphate buffer, did not appear to justify the further use of the more expensive salt.

obtained from a foetus (no. 49) is shown in Fig. 1*b*. The serum was examined about 10 days before term. Only two main components are present, albumin and component 1, which latter must be identical with, or partly composed of, fetuin, the low molecular weight globulin described by Pedersen (1944). The method of extrapolation is obvious from the diagram. Fig. 1*c* presents the pattern of a foetal serum from a Welsh mountain ewe. This serum contains a well-marked component designated as component 2.

Table 4. *Electrophoretic analyses of sheep sera*

Source	Days in lamb	(As percentages of total protein) Albumin	Component 1	Component 2	Buffer	I	pH	Duration of electrophoresis (sec.)	Difference in refraction* $(n_1 - n_0)$
Ram	—	69·8±2	18·0±1	12·2±2	Phosphate	0·2	8·0	14,520	0·00364
Ewe	NP†	36·7±4	24·1±5	39·2±8	Veronal	0·1	8·6	9,120	0·00314
Ewe	58	41·1±2	22·8±3	36·1±2	Phosphate	0·2	8·0	23,100	0·00320
Ewe	58	40·8±2	32·6±3	26·6±1	Phosphate	0·2	8·0	15,920	0·00323
Ewe	95	43·4±4	22·0±2	34·5±2	Phosphate	0·2	8·0	13,920	0·00314
Ewe	112	44·3±0	19·4±3	36·3±3	Phosphate	0·2	8·0	14,460	0·00300
Ewe (defatted)	112	46·7±2	21·8±3	31·5±2	Phosphate	0·2	8·0	17,160	0·00396
Ewe	114	59·1±2	23·0±1	18·0±2	Phosphate	0·2	8·0	21,000	—
Ewe	125	44·5±2	27·6±2	22·8±1	Phosphate	0·2	8·0	16,000	—
Ewe	132	53·6±3	26·2±1	20·2±3	Phosphate	0·2	8·0	30,000	0·00633
Ewe‡	135–138	67·3	21·4	11·3	Phosphate	0·2	8·0	8,820	0·00300§
Ewe‡	137–139	62·6	26·5	10·9	Phosphate	0·2	8·0	13,200	0·00327§
Ewe	146	57·0±2	21·4±2	21·6±1	Phosphate	0·2	8·0	24,000	0·00308

* Dialysed sera: n_1, refraction of inner fluid; n_0, of outer.
† NP means not pregnant.
‡ Stall-fed ewes. The other animals were on pasture.
§ Analysis of cathode side only.

Table 5. *Electrophoretic analyses of foetal sera*

(All the samples were dialysed against phosphate buffer of pH 8 and I 0·2.)

Age of foetus (days)	As percentages of total protein Albumin	Component 1	Component 2	Duration of electrophoresis (sec.)	Difference in refraction† $(n_1 - n_0)$
98*	65·0	23·6	11·4	13,620	0·00317
112*	66·2±3	24·9±5	8·9±1	16,200	0·00414
135	73·0±2	27·0±2	—	13,680	0·00280
135	65·0±4	35·0±4	—	22,300	0·00170
135	77·0	23·0	—	14,220	0·00296
136	69·2	30·8	5·0 (trace?)	30,060	0·00404
134	75·0±3	75·0±3	—	13,440	0·00316

* The mothers (Welsh ewes) of these animals were on pasture; the remainder were stall fed.
† Dialysed sera: n_1, refraction of inner fluid; n_0, of outer.

In Table 4 are presented the results of the electrophoretic analyses of 12 sheep sera from ewes at different stages of pregnancy and one from a ram (Fig. 1*e*). Component 2, expressed as a percentage of the total protein, appears to fall towards the end of pregnancy and there is a corresponding rise in albumin. Further data on this point, preferably from the sera of ewes examined continuously through pregnancy, are desirable. The apparent fall in component 2 at the later stage of pregnancy suggests an association with colostrum formation.

A typical foetal serum electrophoretic pattern

A similar result was obtained from another foetus from a different mother of the same breed.

Table 5 gives the results of the electrophoretic analyses of a number of foetal sera from ewes of two breeds which were on different systems and planes of nutrition. Evidence of the presence of component 2 was found only in the sera from three of the foetuses.

Fig. 2*a* shows the electrophoretic pattern of the serum from a newborn lamb before suckling. This lamb was born from a Welsh ewe and component 2 is present. The patterns for the sera from two 1-day-old lambs are shown in Fig. 2*b*, *c*. Only in the second

Table 6. *Electrophoretic analyses of lamb sera*

Ewe no.	Lamb no.	Age at sampling	Percentages of total protein			Duration of electrophoresis (sec.)	$(n_1 - n_0)$
			Albumin	Component 1	Component 2		
—	757*	15–30 min.	69·0	28·5	2·5	18,960	0·00293
28	102	1 day	65·0±4	25·1±4	9·9±2	10,560	0·00362
53	105	1 „	38·4±1	26·1±12	25·5±1	23,000	0·00297
42	110	4 days	32·5±1	23·6	43·9	13,560	0·00350
30	93	4 „	47·3±3	22·1±4	30·5±2	14,000	0·00354
Ditto defatted		4 „	41·6±3	26·1±1	32·2±1	99,420	0·00227
52	100	4 „	43·9±1	28·4±1	27·6±1	21,840	0·00300
26	88	10 „	62·6	37·4		13,560	0·00280
Ditto defatted		10 „	60·0±2	27·5±1	11·9±2	13,440	0·00280
25	106	10 „	51·8±1	24·5±1	23·7±0	18,000	—
Ditto defatted		10 „	53·5	24·8±2	21·7±2	11,640	0·00356
51	97	10 „	53·8±1	27·1±1	19·1±1	13,260	0·00300
Ditto defatted		10 „	55·5±2	28·3±3	16·1±1	11,640	0·00296

* This lamb had not suckled. All others had suckled normally. Number of feeds about 6–8 for 1-day-old lambs (equal to 500–600 ml. of colostrum or milk).

is component 2 well marked, although it is presumed that suckling had occurred in both cases. Fig. 2*d* is the pattern for a 4-day-old lamb and Fig. 2*e*, *f* for 10-day-old lambs, in each case from a high-plane ewe. Whilst component 2 is well marked in Fig. 2*d*, it is much reduced in the two latter. There is also an anomaly in the latter which is strikingly similar with that described by Longsworth, Shedlovsky & MacInnes (1939) and which, like this, disappears on delipidation (Fig. 2*f*). Fig. 2*g* shows this anomaly also present in the serum of an adult sheep, again disappearing on delipidation, although it is less marked than in the case of the serum of the 10-day-old lamb.

Table 6 gives the details of the electrophoretic analyses of the sera of a series of lambs of different ages. Except for the first lamb they would all have suckled normally. The number of feeds for a day-old lamb would probably be six to eight and equivalent to 500–600 ml. of milk or colostrum.

SUMMARY

1. Results are presented which show that, in the case of pregnant ewes on a high plane of nutrition, their blood sera, obtained approximately 10 days before term, had a higher total protein content than that of ewes on a low plane of nutrition. The same was true of the respective foetal blood sera obtained at the same time. There was no significant difference between the albumin/total protein ratios of the two sets of sera.

2. The same findings hold good for the blood sera of newborn lambs up to 10 days of age, from the ewes fed at high- and low-plane levels respectively.

3. The detailed results of the electrophoretic analysis of some of these sera, as well as those of other ewes, foetuses and lambs, are presented. There was evidence of a fall in component 2 in the sera from the ewes in the latter stage of pregnancy. It is suggested that this may be associated with colostrum formation.

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Iron Metabolism and Haemoglobin Formation in the Embryonated Hen Egg

1. THE OVERALL PRODUCTION OF HAEM PIGMENTS DURING INCUBATION

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Early work on the iron content of hen eggs was reviewed at some length by Needham (1931). The results quoted, however, are very much at variance with one another, and should probably be ignored altogether, because of the inadequacy of the analytical techniques then available. The later work, briefly reviewed by Cruickshank (1941), gives the impression, fully supported by analyses done during the present experiment, that the egg contains on the average about 1 mg. iron, but that there is a large variation between individual eggs. It is agreed that this iron is present almost entirely in the yolk. An observation, first made by Hill (1931), and confirmed by all later workers, showed that practically the whole of the iron present in the unincubated egg reacts with 2:2′-dipyridyl in the presence of a reducing agent to give a pink colour. Since the iron of haem does not give this reaction, it is evident that haem pigments are virtually absent.

It is obvious from the appearance of partially incubated eggs, and from a most superficial consideration of the young chick, that during the 21 days of incubation a large part of the yolk iron is incorporated, presumably after transference to the embryo, into haemoglobin and related pigments. Surprisingly few studies of this aspect of embryo metabolism seem to have been made, and those which have been published are incomplete.

Sendju (1927) washed the blood out of the vessels of 7-, 14- and 19-day embryos and determined the Hb as acid haematin with a Sahli haemoglobinometer. Schønheyder (1938), in the course of an investigation of coproporphyrin metabolism, determined Hb in the 'embryonic areas' of incubated eggs almost daily up to 10 days, and again in 2-day-old chicks which he bled to death. He used the admittedly approximate pyridine-haemochromogen technique of Anson & Mirsky (1929) for his Hb measurements in the embryo. This technique can be expected to determine myoglobin and perhaps other pigments as well as Hb. The data are so scanty that a comparison is scarcely worthwhile, but it is surprising, and by no means explicable on the basis of the perhaps less specific method used by the later worker, that Sendju's figure (23·7 mg.) for Hb in 14-day embryos is very little greater than Schønheyder's (21 mg.) at 10 days. The work described in 'his paper suggests that Sendju's 14-day figure is many times too low. Neither of these workers made any Fe determinations, and it is not quite clear whether either included the membranes in the material analysed. This is a point of no small importance, since both yolksac and chorio-allantoic membrane are richly endowed with blood vessels, and, until the development of the chick is nearly complete, they form a very substantial proportion of the total amount of living matter in the egg.

The accumulation of Fe in the body of the developing chick embryo has been fairly fully studied by McFarlane & Milne (1934), but they did not include the yolksac (and probably other membranous material) in their analyses. They were aware of the error thus introduced, but the picture they gave is incomplete in other respects, since no determinations were made at any stage of the Fe remaining in the yolk. In the liver, but not in the whole chick, they distinguished between total and non-haem Fe, for which they used the method of Hill (1931). These workers were thus able to show a marked accumulation of Fe in the embryonic liver towards the end of incubation. They seem to have found that on the twenty-first day there was about 0·2 mg. non-haem Fe in the liver, which is about one-sixth of the total amount of Fe present in the entire chick. This figure (0·2 mg.) is 10 times greater than that calculated from the authors' figure for total liver Fe, but it is evident from the rest of their analyses and calculations that the liver Fe values which they quote are 10 times too small.

The present work aims at filling some of the gaps in our knowledge of iron metabolism in the embryo. As no direct method for the determination of haemoglobin has yet been found to be reliable when applied to homogenates of whole eggs, determinations have been made, at all stages of incubation, of that proportion of the total egg iron which does not react with 2:2′-dipyridyl in the presence of a reducing agent. Attempts have been made to interpret the results in terms of haem synthesis and haemoglobin production. In this preliminary survey the body of the embryo chick was not separated from its membranes or from the rest of the egg.

EXPERIMENTAL

General. All the eggs used during these experiments were supplied by the Poultry Research Centre, Edinburgh, from their flock of Brown Leghorn hens. The eggs were incubated for various periods under the usual conditions of temperature, humidity, etc. Eggs taken after incubation for 8 days or longer were immersed in acetone-CO_2 for 5 min., stripped of their shells, and homogenized in a glass homogenizer (Potter & Elvejhem, 1936). Eggs incubated for periods less than 8 days burst when immersed in acetone-CO_2. These were therefore frozen in powdered CO_2. The solidified egg was

broken up roughly with a nickel spatula and the still frozen pieces homogenized without the addition of any liquid. For eggs incubated for 18 days or more an equal volume of ice-cold water was added and a preliminary treatment for about 30 sec. in a blendor of the Waring type was used. Careful blendor tests have shown that no iron is introduced into the homogenate from the steel knives of the blendor. The mixture from the blendor was refrozen before glass homogenization. After about the sixteenth day small amounts of feathers and connective tissue form knots resistant to the most vigorous treatment in the glass homogenizer. Such material was discarded. Even in eggs on the point of hatching, it does not amount to more than about 1 g./egg. Sample portions of homogenates prepared in this way were analysed by the techniques described below, and the results obtained used to calculate the amount of Fe (total or non-haem) present in the whole of each individual egg. Each chemical analysis was done in triplicate.

Total Fe. This was determined by a modification of the method previously used for blood (Ramsay, 1944, 1946). The major change was necessitated by the richness of eggs in ether-soluble substances, a fact which makes the homogenate very difficult to digest, except with a very large excess of H_2SO_4-$HClO_4$-HNO_3 mixture. Even though digestion appeared complete, unsatisfactory end points were frequently observed in the final titration. The following technique, of which the unusual feature is the extraction of fatty material with ether before the wet digestion, was therefore adopted. A great economy in the digestion acids, with a consequent reduction in the unavoidable blank is thereby effected, and the end point difficulty seems to have been almost entirely surmounted. Homogenate (1 ml.) is pipetted into the usual small flask, followed by 1 ml. of water and 1 ml. of conc. H_2SO_4 (A.R.). A glass bead is dropped in and the mixture is heated carefully over a small flame until it is apparently homogeneous. The flask is then immersed in boiling water for 45–60 min., during which time a thick, yellow, oily layer of fatty acids separates and floats. After cooling, this layer is removed by extraction successively with 2×3 ml. and 2 ml. of redistilled ether. The ether is removed after each extraction with a very finely tipped Pasteur pipette. The flask is returned to the water bath to evaporate the last traces of ether and after about 20 min. 0·6 ml. of glass-distilled HNO_3 and 0·5 of ml. 60% (w/v) $HClO_4$ (A.R.) are added slowly and very cautiously. The digestion and titration may now be completed as described in the earlier papers, but it is an advantage to leave the flasks in the boiling water bath for about 1 hr. before heating over a free flame. A micrometer syringe burette may be used for the final titanous sulphate titration. It should be noted that only the low concentration of Cu in the egg as a whole (Fe : Cu $\simeq$ 100 : 1, calculated from figures given by McCance & Widdowson, 1940) makes it possible to use the simple direct Ti titration as a measure of Fe.

2:2′-*Dipyridyl* Fe. Hill (1931) added to egg yolk dilute acetate buffer, solid dipyridyl and solid $Na_2S_2O_4$, afterwards making the mixture up to $5\times$ its original volume with ethanol. In the present work it was found that the colour produced with some commercial specimens of $Na_2S_2O_4$ is not stable, and that the error cannot be corrected by the addition of more $Na_2S_2O_4$. Hydroxylamine was therefore employed as reducing agent, in accordance with a recommendation of Jones (1948). Sherman, Elvejhem & Hart (1934) observed that with certain animal tissues full recoveries of Fe could not be attained without using acid of considerably greater concentration than is present in 0·1 N-acetate buffer. Their explanation was that the usual buffer failed to maintain a sufficiently acid reaction. Although this seemed surprising since Hill obtained full colour development up to pH 8, it was thought wise to use an exceptionally concentrated acetate buffer at a pH of about 4·5. The high salt concentration has the additional advantage of reducing to a mimimum the volume of ethanol required to produce a clear solution for the final colorimetric measurement. It may be mentioned that the use of $(NH_4)_2SO_4$ to aid the production of a clear filtrate results in the retention of a high proportion of the ferrous dipyridyl complex with the precipitate. No such effect has been noticed with the acetate concentration finally adopted.

The procedure for all the non-haem Fe analyses quoted here is as follows: 2 ml. egg homogenate is measured into a test tube graduated at 16 ml. To this is added 5 ml. of a solution which is 2 M with respect to Na acetate, 3 M with respect to acetic acid, and which also contains 0·1% redistilled 2:2′-dipyridyl and 0·5% hydroxylamine (in terms of the hydrochloride). The last substance must be virtually freed from Fe by three recrystallizations from the strong acetate buffer containing traces of dipyridyl. After careful mixing, the tube is heated for 10 min. in a briskly boiling water bath, and allowed to cool to about 50°. It is then made up to the mark with ethanol, vigorously shaken, and allowed to cool to room temperature. After a final volume adjustment with ethanol, the mixture is filtered through a Whatman no. 42 paper, and the pink colour is measured in a photoelectric colorimeter, using a green filter with a rather narrow transmission band (Ilford 604 or 624). It is necessary to carry out a blank analysis with a duplicate sample of homogenate, which is put through the whole procedure with a buffer solution free from dipyridyl. All eggs give a yellow colour in the blank estimation, but up to 12 days there is negligible absorption of the light transmitted by either of the above filters. After the twelfth day, however, the blank colorimeter reading gradually increases until at the end of incubation it may be nearly half the total. There is no definite evidence concerning the nature of the substance responsible for the blank reaction; the fact that it absorbs in the green and appears in increasing amount as the Hb rises suggests that the substance may be a haem derivative, but, on the other hand, it first develops at the same time as the down, which in these Brown Leghorn chicks is very highly pigmented. The two Fe methods have been standardized throughout against the same solution of ferric ammonium sulphate.

The difference between total and dipyridyl iron has been assumed to be iron in combination with protoporphyrin IX or some closely related stable substance, and is described here as haem iron. There is a large body of evidence to justify this assumption. On the one hand, it is well known that all but the most stable covalent compounds of iron can be made to react with 2:2′-dipyridyl with the aid, for ferric compounds, of a reducing agent; this has been demonstrated very convincingly by Jones (1948). Although he chose *o*-phenanthroline as his colour-developing agent, it seems from his paper that closely similar results are obtained with dipyridyl. It was not necessary to make any further check on this aspect of the test, but since most of the older

dipyridyl techniques do not involve heating, it has been thought wise to prove the non-reactivity of haem iron. When a preparation of haemin containing 8·42 % iron (determined by titanium titration after wet digestion) was put through the dipyridyl procedure described above, only 0·18 % of the total iron was found to react. This result is conclusive, as far as haem is concerned, and the reasonably close agreement between the two methods, when both are applied to unincubated eggs, confirms the absence from such material of any other iron compounds which might not react with dipyridyl.

RESULTS AND DISCUSSION

The two analytical techniques described above have been applied to homogenates prepared from a total of 115 eggs incubated for various periods between

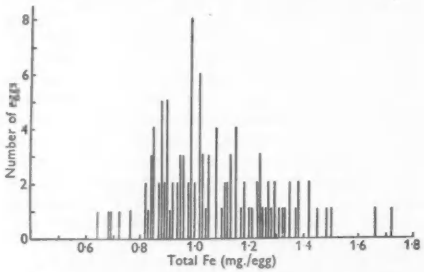

Fig. 1. The total iron content of 115 Brown Leghorn eggs.

0 and 21 days, and before discussing the results in detail it seems desirable to emphasize the considerable variations which have been found in the iron content of the eggs. Since unpublished observations in this laboratory show variations of similar magnitude in other egg constituents, caution should be exercised in the interpretation of all experiments based on small numbers of eggs. The iron content of the eggs used in the present work averaged 1·067 mg. per egg, with a standard deviation of ± 0·20 mg. and an extreme range of 0·64–1·72 mg. per egg. The values were approximately normally distributed, as may be seen from Fig. 1. No relation between the size of the egg and its iron content was noticed, as very large eggs frequently have quite low iron concentrations and vice versa; but individual variation is so great that it might be necessary to analyse some hundreds of eggs before definite conclusions could be drawn.

Twelve unincubated eggs were analysed for both total and non-haem iron. The proportion of non-haem iron ranged from 95 to 101 % of the total, with a mean of 98 % (S.D. ± 1·5 %). These results are in close agreement with those of previous workers, e.g. Hill (1931), and justify broadly the analytical techniques. The present procedure is, however, quite inadequate for detecting, let alone determining, the small but definite quantity of haem which is synthesized during the early days of incubation; already at the third day blood can be seen in the vessels of the yolk sac with the naked eye, but not until about the sixth day is the analytical difference between total and non-haem iron significant. By about the tenth day the difference probably provides a fairly accurate estimate of the amount of haem iron present.

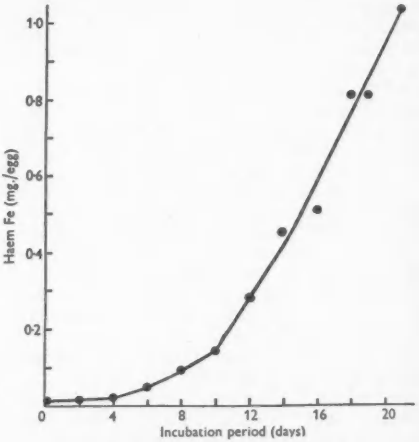

Fig. 2. The increase of haem iron during the incubation of eggs.

Fig. 2 shows the progress of the haem iron content, in mg. per egg, throughout the course of incubation. All the points on the figure, except that for the twenty-first day, represent the means for four to eight eggs. The last point represents only two eggs; this is chiefly because it is very difficult to make eggs, on the point of hatching, into a true homogenate, without running the risk of introducing extra iron. These two eggs, however, were both well chipped at the moment they were frozen, and had thus reached the same stage of development. Moreover, the results agree closely with one another, and as the point fits in so neatly with the others there seems to be no reason to doubt its validity. This justification applies more particularly to the calculations of haem iron as a percentage of the total iron which are shown in Fig. 3. It will be seen from Fig. 2 that haem synthesis proceeds comparatively slowly until the eighth day, when the egg contains about 0·08 mg. haem iron, and the curve then steepens markedly, so that at 10 days the value for haem iron is about 0·15 mg., at 14 days 0·42 mg., at 18 days 0·76 mg. and at hatching 1·03 mg.

The mean haem iron content of the later eggs exceeds considerably the total iron content of some of the individual eggs used in the investigation. This observation has prompted the construction of Fig. 3, showing the proportion of the total iron present at any given stage of incubation as haem iron. From the twelfth day onwards this proportion was remarkably constant for eggs of equal incubation

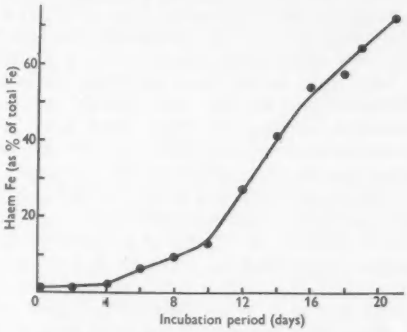

Fig. 3. The increase of haem iron, expressed as a percentage of the total iron, during the incubation of eggs.

periods, and the whole figure throws a new light on the matter. Thus, between the sixth and the tenth day about 2% of the total iron is incorporated into haem each day. From the tenth day at least until the sixteenth day the rate of synthesis expressed in this way is nearly constant, but about three times as great: something over 6% of the total being transformed into haem iron each day. During the last 5 days there is a slight fall in the rate, but this may be more apparent than real. There is at this stage some formation of bile pigments which probably means that some breakdown of haem is taking place; the quantities of haem observed in the present experiments must be, of course, the resultants of synthetic and destructive processes.

The fact that during the last 8 or 9 days of incubation the haem iron forms a relatively constant proportion of the total is worthy of further comment. This applies to all the later groups of eggs, but a series of 18-day eggs with a particularly wide range of total iron contents illustrates the point particularly well. In Fig. 4 the haem iron is plotted against the total iron for each of the eight 18-day eggs. It is seen that the amount of haem increases with the total iron, and that the points for the individual eggs lie quite close to the straight line calculated from Fig. 3, using the equation: Haem-Fe = 0·59 × total Fe. Thus in the embryonated egg, as in so many other biological systems, the quantity of iron available may be one of the limiting factors in haem synthesis. Whether it has an appreciable influence on the development of the embryo or the hatchability of the egg is at present uncertain. The following observation is, however, at least suggestive: of the 115 eggs used for these experiments 37, or 32·2%, contained 0·95 mg. iron or less, while of the thirty-eight eggs out of the total which were incubated for 14 days or longer only four, or 10·5%, were low in iron to this extent. These figures would be consistent with the hypothesis that embryos in eggs particularly low in

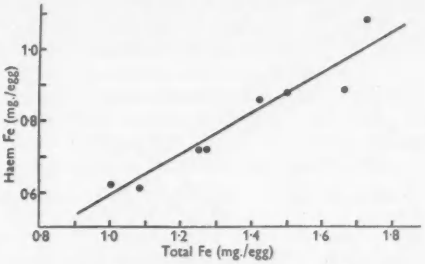

Fig. 4. The relation of haem iron to total iron in eggs incubated for 18 days.

iron tend to die during the first fortnight of incubation. The analysis of unsuccessfully incubated eggs has not yet been undertaken, but may provide an answer to this important question.

Comparison of the amount of haem pigment in the egg with the total weight of the embryo

No observations on embryo weight were made during the present investigation, and the technical skill required for making reliable measurements of the weight of the membranes was not available, but figures based on more than 2000 eggs are given by Byerly (1932). As the small number of embryo weights recorded in the present work agree quite well with those of Byerly, it was considered safe to use his figures, especially as the comparisons involve not absolute weight but the proportion of the final weight reached at different stages in incubation. In Fig. 5 the period of incubation, from the tenth until the nineteenth day, is plotted separately against the haem iron, the embryo weight excluding membranes, and the embryo weight including the membranes, each calculated as a percentage of its magnitude at hatching. The haem values have been calculated on the basis of the analytical observation that at 21 days 72% of the total iron in the egg is present as haem. Although it is true that a larger number of eggs might have given a mean percentage slightly different from this, the general trend in Fig. 5 would not have been altered. The embryo and membrane figures have been calculated from Byerly dry weights. As the body of the chick becomes progressively 'less wet' during the latter half of incubation, somewhat

different figures are obtained if wet weights are used, but again the general trend of the results is the same. Comparison of the graphs shows that a much larger proportion of the final amount of haem than of the gross body weight has been manufactured during the earlier stages of incubation. Thus, at 12 days, 37 % of the final amount of haem has been accumulated, whereas the body of the chick has only reached 10 % of its final weight. At 15 days about 63 % of the final amount of haem has made its appearance, but the chick-body weight is still only 40 % of its final value. By 18 days the two values

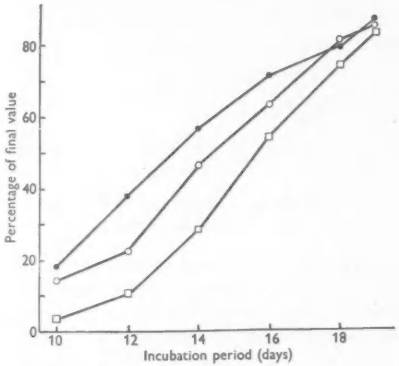

Fig. 5. The relation of haem synthesis to chick embryo growth. ●——●, haem content as a percentage of its final value; □——□, embryo dry weight as a percentage of its final value (Byerly, 1932); ○——○, embryo + membranes, dry weight, as a percentage of their final value. All these functions are plotted against the period of incubation.

are respectively 81 and 74 %, while a day later they are even closer at 87 and 83 %. At 21 days the values must of course both be 100 %; what requires emphasis is that at certain stages of development the rate of haem synthesis and hence almost certainly the rate of haemoglobin manufacture is relatively much higher than that of gross growth. The most probable explanation is suggested by the appearance of the egg, with its extensive yolk sac and allantoic membrane, both obviously richly supplied with blood: except towards the end of incubation, when these membranes disappear, a large part of the egg haemoglobin cannot be in the body of the chick at all, but must be circulating in the membranes. That this is at least partially true is shown by the middle curve in Fig. 5, which describes the change in embryo weight including the membranes, again calculated at each stage as a percentage of the final value. This line lies a good deal closer to the haem graph than does that for embryo weight alone, and the difference that still remains may well be largely accounted for by the fact that the membranes are organs of a rather uncommon shape, with a blood supply which, relative to their bulk, is unusually copious. The foetal placenta may perhaps impose similar and rather high demands on the haem-synthesizing mechanism at certain stages of the development of the mammalian embryo.

Although all attempts to make direct haemoglobin determinations on egg homogenates with a precision at all approaching that of these iron methods have so far been unsuccessful, it is possible to calculate approximate haemoglobin figures from the haem iron estimations. Drabkin (1948) has published figures for the total content in several mammalian species of the three chief haem compounds: haemoglobin, myoglobin and cytochrome *c*. In the adult rat haemoglobin amounted to 92 % of the total, in man 96 %, and in the cow 88 %. In the horse the proportion was somewhat lower, only 75 %, chiefly because of the presence of an unusually large quantity of myoglobin. Although a comparison between adult mammals and embryo chicks is somewhat hazardous, it seems likely that no great error will result from the assumption that 90 % of the haem in the egg is present in haemoglobin. Such a figure might be too high in the earliest stages of development, but these are not considered here, and might again be too high towards hatching time, since no information is available concerning the amount of myoglobin in the muscles of the unhatched chick. The error is unlikely to be greater than that involved in rinsing from embryo or chick vessels a fluid which clots so very readily as chicken blood. Table 1 summarizes haemoglobin figures from the papers of

Table 1. *Haemoglobin content of eggs and embryos*

(mg. Hb/egg or/embryo)

Age (days)	Embryos (Sendju, 1927)	Embryonic areas (Schønheyder, 1938)	Whole eggs (This work)
6	—	4·3	—
7	7·6	—	—
8	—	15·7	26
10	—	21	43
14	23·7	—	110
19	141	—	185
21	—	—	208
2-day-old chicks	—	173	—

Sendju (1927) and Schønheyder (1938), and from the present work, calculated on the assumption which has just been discussed and the additional one that embryonic chick haemoglobin, like that from so many other sources, contains about 0·335 % iron. The calculations have been made from the values in Fig. 3 for the proportion of the total iron present as haem iron, and from the general mean value of

1·07 mg. total iron per egg. The present estimates are at all times higher, sometimes very much higher, than the previous ones, probably because of the inclusion of all the haemoglobin-containing material in the egg. Schønheyder believed that his figure for the total haemoglobin content of 2-day-old chicks might be low, and if that is taken into account his value is in good agreement with the present one. A rough estimate of the haemoglobin content of the hatching chick is about 200 mg., although of course it is clear from Fig. 4 that there must be large variations among different individuals.

SUMMARY

1. A modification of the wet-digestion titanium-technique for total iron, suitable for application to eggs, is described. Total iron in 115 eggs has been found to average 1·07 mg. per egg.

2. A modification of the 2:2′-dipyridyl method for the determination of non-haem iron is described. It is confirmed that almost the whole of the iron in unincubated hen eggs is non-haem iron.

3. The increase in haem iron during the course of incubation is described. It amounted to 0·14 mg. per egg after 10 days' incubation, 0·41 mg. at 14 days, and 1 mg. at 21 days.

4. During the last 9 days of incubation a fairly constant proportion of the total iron is present as haem iron at any given stage of development. This increases from 40 % at 14 days to 72 % at 21 days.

5. Haem synthesis is discussed in relation to: (i) the total amount of iron present, and (ii) the growth of the embryo and its membranes.

6. Approximate haemoglobin values are calculated from the haem iron figures. It is concluded that the average egg on the point of hatching probably contains about 200 mg. haemoglobin.

The author is indebted to Prof. G. F. Marrian, F.R.S., for constant encouragement; to the staff of the Poultry Research Centre, Edinburgh, for their co-operation in the supply and incubation of eggs and to Mr D. G. McLeod for valuable technical assistance.

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Studies on Pituitary Adrenocorticotrophin

1. ULTRAFILTRATION OF THE HORMONE

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Smith (1926) demonstrated that the adrenals of the rat undergo atrophy following hypophysectomy. He also showed (Smith, 1930) that the anterior lobe of the pituitary gland contained a factor which could bring about the reversal of this adrenal atrophy. He further found that both atrophy and repair were limited to the adrenal cortex. The factor has since been termed the adrenocorticotrophic hormone. Later, several workers attempted to isolate and characterize the hormone (Collip, Anderson & Thompson, 1933; Collip, 1938; Lyons, 1937; Bates, Riddle & Miller, 1940). These investigations suggested that the hormone was protein or polypeptide in nature, but differed from the other anterior pituitary hormones in its remarkable thermostability.

Sayers, White & Long (1943) and Li, Evans & Simpson (1943) simultaneously published details of the preparation, from pig and sheep pituitaries respectively, of proteins of high adrenocorticotrophic potency which appeared to be homogeneous by the criteria of sedimentation, electrophoresis and constant solubility. The preparations from pig and sheep glands, and also material prepared by a

similar method from ox glands (White, 1944), appeared to be identical in biological, chemical and physical properties. Molecular kinetic data indicated a molecular weight of 20,000.

While the characterization of these protein hormone preparations appears satisfactory, there is considerable evidence indicating that the biologically active principle may be of much smaller molecular size. The thermostability suggests that the particular grouping in the protein hormone which is essential for biological activity may be relatively small, and may not involve the whole surface of the molecule as is the case for many other protein hormones, such as insulin. This conclusion is supported by the observations of Li *et al.* (1943) who were able to hydrolyse 38 % of the protein hormone with pepsin without affecting the biological activity. Later Li (1947) partially hydrolysed the protein hormone with hydrochloric acid and found adrenocorticotrophic activity in the hydrolysates.

Anselmino, Hoffmann & Herold (1934*a*, *b*) claimed to have ultrafiltered adrenocorticotrophin through a collodion membrane. Their claim has been regarded with some doubt by later workers on account of the lack of specificity of their bioassay method. Tyslowitz (1943), using adrenal hypertrophy in hypophysectomized rats as his method of assay, also reported the ultrafiltration of the hormone through cellophan. Acetic acid extracts of acetone-dried gland powder (pig, sheep or ox pituitaries) were used in this work. About 50 % of the solids passed through the membrane accompanied by 38 % of the biological activity. The ultrafiltrate solids were, therefore, slightly less active than the starting material. This may be partly accounted for by inactivation, since approximately 30 % of the initial activity was lost. The relatively large amount of solids passing the membrane is noteworthy and suggests that the membranes used were of comparatively high permeability.

Sayers *et al.* (1943), in the course of their preparation of the adrenocorticotrophic protein, dialysed their protein fractions. A considerable portion of the total biological activity of the extract appears to have been lost in this stage. Sayers, Sayers & Woodbury (1948) report that an Armour preparation of adrenocorticotrophin (no. La-1-a) which was heterogeneous by electrophoresis was significantly more active biologically than an electrophoretically homogeneous preparation made by the method of Sayers *et al.* (1943).

In 1938 we attempted to repeat the work of Anselmino *et al.* (1934*a*, *b*) using adrenal hypertrophy in the hypophysectomized rat as our method of assay. We were unable to reproduce their results, but, after some modifications, succeeded in preparing ultrafiltrates of high biological activity. It was evident that the optimum conditions for ultrafiltration were critical, and a more thorough study of these conditions was necessary. The results of this work are given in the present paper. A preliminary communication of the work has already been presented (Crooke, Henly & Morris, 1947).

EXPERIMENTAL

Bioassay methods. The majority of the bioassays in this work were carried out by the adrenal hypertrophy method in the hypophysectomized rat. The method used was based on the results of Crooke & Gilmour (1938).

Male rats of Wistar strain were hypophysectomized at 35–42 days (90–110 g. body weight); 10–14 days after hypophysectomy, subcutaneous injections of the test material were given twice daily for 3 days, four animals being used at each level. The rats were autopsied on the fourth day, adrenals and testes dissected free of extraneous tissue and weighed. Testes weight was taken as a criterion of completeness of hypophysectomy, animals having a testes weight greater than 500 mg. being discarded. In practice, testes weights were usually below 400 mg. In some cases the adrenals were examined for histological evidence of repair (Simpson, Evans & Li, 1943). Adrenal weights are expressed in mg./100 g. body weight. The body weight used is that at the commencement of the injections. One adrenocorticotrophic unit is arbitrarily defined as a 50 % increase in mean adrenal weight over that of hypophysectomized controls. The probable range of adrenal weights for fifty normal rats was $20{\cdot}7 \pm 5{\cdot}8$ mg./100 g. body weight (mean $\pm$ twice standard deviation). The adrenal weights for hypophysectomized controls were 8–10 mg. A typical set of assay results is given in this paper which demonstrates that, by careful attention to strain and nutritional status of the animals used, a satisfactorily linear relation can be obtained between the logarithm of the dose and the increase in adrenal weight.

Some preparations have been assayed by the adrenal ascorbic acid depletion method of Sayers *et al.* (1948). The use of pentobarbital anaesthesia for adrenalectomy and intravenous injection of the test material gave very variable responses in our strain of rats. Substitution of ether anaesthesia gave reliable results.

Preliminary extraction and fractionation. Ox pituitary glands were collected in batches of 200 in a mixture of 10 % methanol and 90 % ethanol immediately after slaughter, and subsequent stages were carried out with a minimum delay. The glands were freed from the ethanol mixture, frozen with solid CO_2, the anterior lobes separated and ground mechanically with powdered CO_2. Acetone (2·5 l.) was added to the mixture which was then allowed to warm up to room temperature. The powdered gland material was separated by vacuum filtration and air-dried for 12 hr. Yield from 200 glands is 60–80 g. The subsequent fractionation and distribution of biological activity are shown in Fig. 1. All manipulations were carried out at room temperature.

The experimental details are illustrated by the following example. Acetone-dried pituitary powder (70 g.) was stirred with 2·1 l. 0·25 % (w/v) NaOH until solution was complete (1–1·5 hr.). The mixture was adjusted to pH 5·5 (all pH values measured with the glass electrode) with glacial acetic acid, and, after stirring for a further 15 min., was centrifuged. The precipitate was stirred for 1 hr. with 1 l. 70 % (v/v) ethanol to which sufficient concentrated HCl (*d* 1·18) had been added to give a final pH of 2·2–2·3. The mixture was

centrifuged for 15–30 min. It was often difficult to get good separations at this stage. The insoluble material was re-extracted three times in a similar manner using 750, 500 and 250 ml. portions of 70% ethanol at pH 2·2–2·3. The combined extracts were adjusted to pH 5·5 with 10N-NaOH and stored at 0° overnight. Next day the precipitate was centrifuged, washed twice with acetone and dried in air and *in vacuo*. The resulting solid (fraction *B*) was ground mechanically to a fine powder and stored *in vacuo* at 0°. The biological activity was unchanged after 2–3 months' storage under these conditions. The yield was variable, 10–18 g. from 200 glands. This is probably accounted for by the difficulty of clarifying the acid ethanol extracts. 60–75% of the biological activity of the acetone-dried powder was recovered in this fraction with 10–20% of the solids. Preparations of protein hormone were made from pig and ox glands by the method of Sayers *et al.* (1943), as modified by Fishman (1947).

The membranes used for routine purposes were of grade 300 cellophan which had been soaked in distilled water for at least 6 hr. prior to use.

The average pore diameter of these membranes is quoted by the makers (British Cellophane Ltd., 17–19 Stratford Place, London, W. 1) as 3·0 mμ. Determination by the method of Elford & Ferry (1935) gave a value of 3·4 mμ. Since the assumptions on which this method is based are of doubtful validity for membranes of low porosity (Elford & Ferry, 1935; Ferry, 1936), the permeability of the membrane under our experimental conditions to proteins of known molecular weight was investigated. The membrane was impermeable to horse myoglobin (mol.wt. 17,500) and crystalline ribonuclease (mol.wt. 13,700), but was permeable to salmine (mol.wt. 8000).

The mixtures for ultrafiltration were prepared by stirring 1–3 g. of fraction *B* with the appropriate solvent to give a 4% (w/v) solution. Stirring was continued for 1–1·5 hr. When acetate buffers were used, fraction *B* was stirred with the dilute alkali until solution was complete and the mixture adjusted to the indicated pH with acetic acid. The results are shown in Table 1.

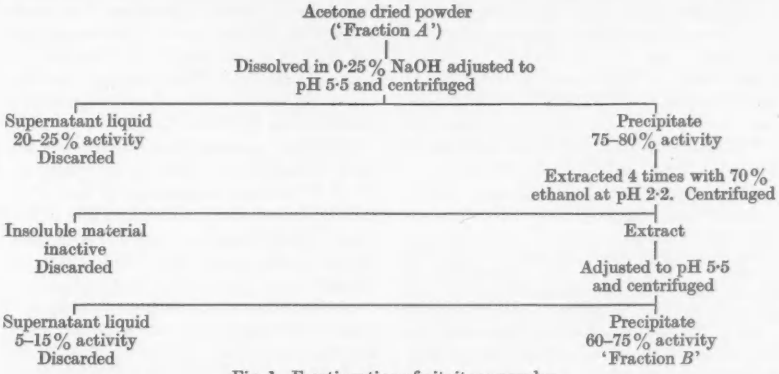

Fig. 1. Fractionation of pituitary powder.

Ultrafiltration. The process was carried out in a metal ultrafilter lined with 'Tufnol' plastic. The effective ultrafiltration area was 8·1 sq.cm. (area of holes in the perforated plate supporting the membrane). The volume of liquid ultrafiltered should not exceed 10 ml./sq.cm. effective ultrafiltration area. Ultrafiltration was carried out under N_2 at 6 atm. pressure.

Activities are expressed in adrenocorticotrophic units per gland. N analyses were carried out by the micro-Kjeldahl

Table 1. *Comparison of ultrafiltrations in different media*

Ultrafiltration medium	Before ultrafiltration			Ultrafiltrate			Recovery in ultrafiltrate		
	Solids (mg./ml.)	N (mg./ml.)	Activity (units/gland)	Solids (mg./ml.)	N (mg./ml.)	Activity (units/gland)	Solids (%)	N (%)	Activity (%)
2M-Na acetate-acetic acid, pH 3·9	40·0	5·23	1·8	31·3	0·09	0·7	—	1·72	38·9
0·1M-Na acetate-acetic acid, pH 3·9	40·0	5·28	1·8	11·2	0·06	0·4	—	1·14	22·2
0·1M-Na acetate-acetic acid, pH 5·04	44·0	5·89	1·7	9·2	0·03	0·3	—	0·51	17·6
0·1M-Na acetate-acetic acid, pH 6·06	44·0	5·89	1·7	10·5	0·03	0	—	0·51	0
0·1M-Na acetate-NaOH, pH 9·8	44·0	5·78	1·7	11·9	0·06	0·3	—	1·04	17·6
0·1M-Li acetate-acetic acid, pH 3·9	40·0	5·56	1·8	8·3	0·06	0·4	—	1·08	22·2
0·1N-HCl, pH 0·6	44·0	5·89	1·7	1·4	0·12	0·6	3·18	2·04	35·3
1N-Acetic acid	40·0	5·40	1·8	1·5	0·10	0·9	3·76	1·85	50·0
2N-Acetic acid	64·0	8·52	1·7	2·0	0·16	1·1	3·13	1·87	64·7
4N-Acetic acid	64·0	8·52	1·7	2·8	0·25	1·1	4·30	2·93	64·7

method. Fig. 2 shows the effect of pH on the percentage recovery of biological activity in the ultrafiltrates. The broken line indicates the limit of significance of the bioassay at $P=0{\cdot}04$.

The biological activities at various stages in the process are shown for two typical preparations AH 30 and AH 35 in Table 2.

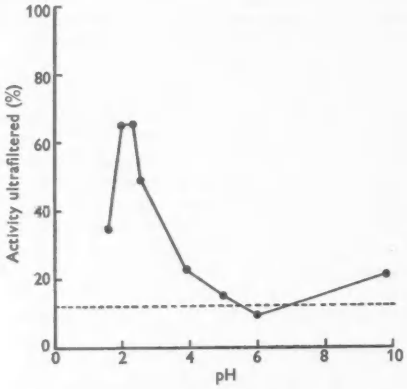

Fig. 2. The effect of pH on the efficiency of ultrafiltration.

Ultrafiltration experiments were also carried out with a 'crude prolactin' fraction prepared by the method of White, Bonsnes & Long (1942). All the activity of the preparation passed through the membrane, but the relatively small proportion of the initial biological activity recoverable in the 'crude prolactin' fraction rendered the method unsuitable for preparative purposes.

and contain 1·5–2·7 mg. total solids per ml. Ash content averages 20% of the total solids and nitrogen content 10% of the organic matter. The ultrafiltrates may be lyophile-dried without loss of activity, to give a colourless powder. This gives positive biuret, ninhydrin, tyrosine (Folin-Ciocalteu reagent) and Sakaguchi reactions. The Pauly diazo reaction for histidine was negative. Examination of hydrochloric acid hydrolysates by partition chromatography on paper (Consden, Gordon & Martin, 1944) revealed the presence of the following amino-acids: glycine, alanine, valine, leucine, and/or isoleucine, glutamic acid, aspartic acid, methionine, tyrosine, phenylalanine, proline, arginine, lysine, serine and threonine. Paper partition chromatography of the original material in phenol-0·1% cupron revealed the presence of at least ten components as distinct spots. The biological activity of the ultrafiltrates is stable for 1 hr. at 100° in 0·1N-hydrochloric acid. Preliminary experiments with papain indicate that the activity is destroyed by digestion for 12 hr. at 37°.

Biological activity. The biological activity of the total ultrafiltrate solids of a typical preparation, no. AH 44, was compared with that of the Armour standard adrenocorticotrophin preparation, no. La-1-a, by the adrenal ascorbic acid depletion method of Sayers *et al.* (1948). The assay data are given in Table 3.

Statistical analysis of these data by the method of Irwin (1937) shows that the logarithm of the ratio of the potency of AH 44 to La-1-a is 0·070, the standard error of $M(S_M)$ is 0·056 and the combined standard

Table 2. *Concentration and recovery of biological activity during fractionation*

	AH30			AH35		
Fraction	Activity (units/g.)	Activity (units/mg. N)	Total activity recovered (%)	Activity (units/g.)	Activity (units/mg. N)	Total activity recovered (%)
Acetone-dried powder	7·0	0·08	100	7·2	0·07	100
Fraction *B*	35·8	0·24	77	19·4	0·15	60
Total solids of 2N-acetic acid ultrafiltrates	520	6·8	46	420	5·3	39
Enrichment factor	74	85	—	58	76	—

A preparation of protein hormone made from whole pig pituitary glands by the method of Fishman (1947) was assayed by the adrenal hypertrophy method of Sayers *et al.* (1943) which differs from our own method only in the use of three intraperitoneal instead of two subcutaneous injections per day. A total dose of 5 mg. per rat produced a mean adrenal weight of 17·7 mg./100 g. body weight. Ultrafiltration of this preparation in 0·1M-Na acetate-acetic acid at pH 3·9 resulted in complete recovery of the biological activity in the ultrafiltrate.

Chemical properties of ultrafiltrates. Ultrafiltrates obtained under the optimum conditions using 2N-acetic acid as ultrafiltration medium are colourless

deviation (σ) is 19·7. AH 44 is, therefore, 1·180 times as potent as La-1-a and the estimate of potency will lie between 1·29 and 0·977 in 95 trials out of 100. Since Sayers *et al.* (1948) found La-1-a to be 1·185 times as potent as an electrophoretically homogeneous preparation of protein hormone made by the method of Sayers *et al.* (1943), AH 44 is 1·40 times as potent as the latter. AH 44 was also assayed by the adrenal hypertrophy method in hypophysectomized rats. Assay data are given in Table 4 and Fig. 3. *P* values are comparisons between injected animals and hypophysectomized controls. The weights given are means $\pm 2 \times$ standard deviation.

The linear relation between logarithm of dose and adrenocorticotrophic potency is shown in Fig. 3. The mean activity of AH 44 is 0·468 unit/mg. The preparation of protein hormone prepared from pig pituitaries by the method of Sayers *et al.* (1943) had an activity of 0·288 unit/mg. While the data are insufficient to permit of accurate comparison, the potency ratio of 1·62 is in fair agreement with the value of 1·40 derived from the ascorbic acid depletion assay. No correction has been made for the inorganic content of AH 44, which yielded ash equivalent to 19·2 % of the dry weight.

Table 3. *Comparison of biological activities of protein and ultrafilterable hormones by the adrenal ascorbic acid depletion method*

Preparation	Dose (μg./100 g. body weight)	No. of animals	Mean depletion of ascorbic acid (μg./100 mg. adrenal tissue)
La-1-a	0·25	6	22·8
	0·50	6	65·1
	1·00	4	113·0
AH 44	0·25	4	33·0
	0·50	4	79·3
	1·00	4	117·0

Table 4. *Assay of ultrafilterable hormone by the adrenal repair method*

Dose (mg.)	No. of animals	Mean adrenal weight (mg./100 g. body wt.)	*P*
0	7	7·9±3·0	—
2·5	4	14·1±5·6	<0·07
5·0	4	18·8±3·8	<0·01
7·5	4	21·4±4·8	<0·01
10·0	4	23·5±3·6	<0·01

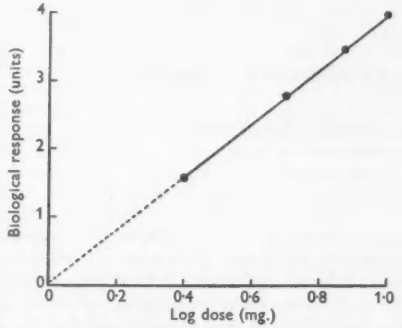

Fig. 3. Relation between dose administered and adrenocortical hypertrophy in hypophysectomized rats.

The ultrafiltrates are devoid of luteinizing or follicle-stimulating activities. Oxytocic activity was less than 0·006 i.u./mg.

DISCUSSION

The results of the ultrafiltration experiments given in Table 1 indicate that the best recoveries of biological activity are obtained in acetic acid solutions of normality 2·0 or greater. Ultrafiltrates from 4N-acetic acid are no more active than from 2N, but the total solids and nitrogen in the ultrafiltrates are greater. In subsequent experiments 2N-acetic acid has, therefore, been adopted. The course of the fractionation shown in Table 2 shows that about 40 % of the original activity of the acetone-dried gland powder was recovered in the ultrafiltrates, and was concentrated at least 60-fold. The efficiency of the ultrafiltration stage (55–65 %) is undoubtedly lowered by blocking of the membrane pores by non-ultrafilterable proteins. The efficiency can be increased by working at lower protein concentrations, but the increased duration of ultrafiltration makes the gain of doubtful value. Further fractionation of fraction *B* prior to ultrafiltration would probably increase the efficiency of the latter process, and such fractionation is now being investigated.

The relatively high recovery of the initial biological activity, and the fact that the biological activity of the protein hormone of Sayers *et al.* (1943) is itself ultrafilterable, makes it unlikely that there is more than one adrenocorticotrophic factor (measured by adrenal hypertrophy and depletion of adrenal ascorbic acid) in the pituitary extracts, as suggested by Li (1947). Since a protein of molecular weight 20,000 cannot pass through the membranes used in this work, it seems probable that the protein hormone can be dissociated under certain conditions into smaller units, one or more of which may carry the biological activity. This view is supported by the results presented in Fig. 2. The marked pH specificity of the ultrafiltration process suggests dissociation of electrovalent linkages, the low recovery at pH's 0·6 and 6·06 being particularly striking. It appears unlikely that disulphide linkages are involved in the dissociation since the addition of reducing agents to the ultrafiltration medium did not increase the efficiency of the process. The fact that the total ultrafiltrate solids are at least 1·4 times as active as the protein hormone also suggests that the biological activity of the latter may be contained in a relatively small grouping. Various fractionation studies on the ultrafiltrates, particularly frontal analyses and displacement development on carbon columns (Tiselius, Morris & Morris, 1948), indicate that the active principle constitutes not more than 10 % of the total ultrafilterable solids. The passage through cellophan membranes indicates that the molecular weight of the ultrafilterable hormone must be less than 13,000. The ratio of amino to total nitrogen in ultrafiltrates indicates an average value of 8–10 amino-acid residues per peptide molecule. Li (1947) found

biological activity in partial hydrolysates of the protein hormone which had an average of 7–8 amino-acid residues per peptide molecule. The results of the papain hydrolysis suggest that at least one peptide link is necessary for biological activity. The physiological significance of the dissociation of the adrenocorticotrophic protein, especially as regards its release from the pituitary cells into the circulation and its entry into the target organs, the adrenals, must await further work.

SUMMARY

1. The fractionation of ox anterior pituitary glands with a view to the concentration of the adrenocorticotrophic hormone is described.

2. The conditions for ultrafiltration of the biological activity have been investigated, and ultrafiltrates of high potency obtained.

3. The total biological activity of the protein hormone prepared by the method of Sayers *et al.* (1943) has been shown to be ultrafilterable under these conditions.

4. The chemical and biological properties of the ultrafiltrates are described.

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Determination of β-Naphthoxyethanol in Blood

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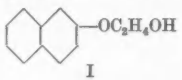
β-Naphthoxyethanol (I) has been shown by Francis & Parry (1949) to be a useful short-acting anaesthetic particularly for the horse. It was desired to correlate the pharmacological action of this compound with



I

its concentration in the blood, and to study its absorption and excretion. Attempts were therefore made to develop a method of determining it in biological material. At first potential colorimetric methods were examined. Nitration of β-naphthoxyethanol, followed by reaction of the polynitro compound with butanone in the presence of alkali, gave a red colour; but this was unstable, and its intensity was greatly influenced by small variations in the conditions of nitration. The possibility of hydrolysing the drug to β-naphthol and determining the latter by reaction with diazonium salts did not appear more promising. However, it was found that β-naphthoxyethanol shows selective and characteristic light

absorption in the ultraviolet and the following method of determining it is based on this property. The conditions described have been applied to numerous other aromatic drugs showing selective ultraviolet absorption.

EXPERIMENTAL

Absorption spectrum of β-naphthoxyethanol. β-Naphthoxyethanol was purified by three recrystallizations from light petroleum (b.p. 40–60°) and dried at 60° *in vacuo* for 1 hr. (m.p. 75°). The ultraviolet absorption spectrum of a 0·0005% solution in $CHCl_3$ (w/v) was examined in a 10 cm. silica cell in the Beckman quartz spectrophotometer, model DU, and is shown in Fig. 1; in Table 1 maxima, minima and inflexions

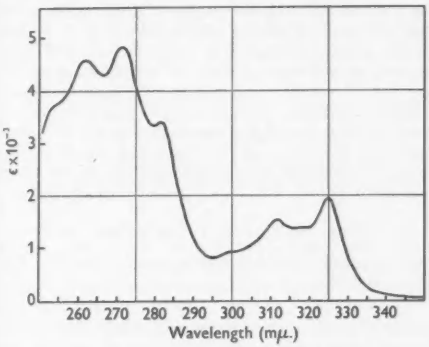


Fig. 1. Absorption spectrum of β-naphthoxyethanol in chloroform.

are summarized. The maximum at 262 mμ. was thought to be unsuitable for study because of the nearness of this wavelength to the region of critical sensitivity of the Beckman instrument. The maxima at 272 and 325 mμ. were therefore selected. The validity of Beer's law at these wavelengths for solutions of β-naphthoxyethanol ranging in concentration from 0·05 to 0·5 mg./100 ml., and the linearity of instrument response within this range were established (Fig. 2), and showed that solutions of β-naphthoxyethanol in $CHCl_3$ could be accurately analysed by comparison with a single standard. The effect of varying slit width at both wavelengths was negligible (Fig. 3). It was decided to analyse unknown solutions by determining the optical densities at both 272 and 325 mμ., and to take the average of the two results, since this would enable the identity of β-naphthoxyethanol, isolated from biological material, to be checked, and also increase the accuracy of analysis.

Extraction. Preliminary experiments on the isolation of β-naphthoxyethanol from blood and urine showed that a single extraction with $CHCl_3$ removed it quantitatively, but also that normal blood and urine did not give transparent

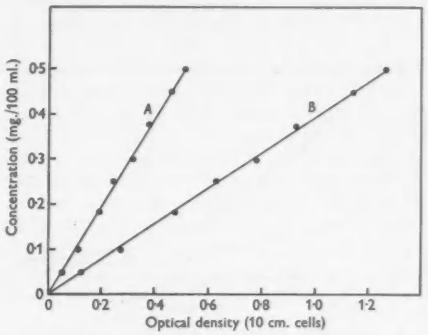


Fig. 2. Optical density of chloroform solutions of β-naphthoxyethanol at 272 (*B*) and 325 (*A*) mμ.

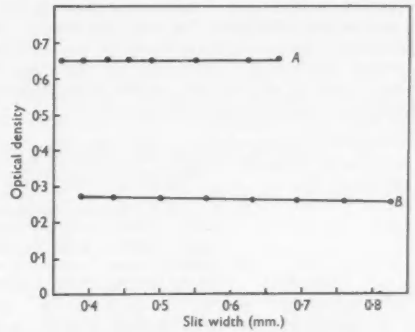


Fig. 3. Variation of optical density of a chloroform solution of β-naphthoxyethanol with change in slit width, at 272 (*A*) and 325 (*B*) mμ.

extracts. Fig. 4 shows the ultraviolet absorption of chloroformic extracts of 2 ml. of blood or 2 ml. of urine. The absorption of both extracts, although non-specific, was not negligible. Attempts were made to reduce this absorption by varying the pH at which the extraction was performed (Table 2), but the effect was small, some slight advantage being achieved by extracting from a basic medium. The extraction was cleanest using Na_2HPO_4, which was therefore

Table 1. *Maxima, minima and inflexions from the ultraviolet absorption spectrum of β-naphthoxyethanol*

Maxima			Minima			Inflexions		
λmμ.	$E^{1\%}_{1\,cm.}$	ε	λmμ.	$E^{1\%}_{1\,cm.}$	ε	λmμ.	$E^{1\%}_{1\,cm.}$	ε
262	243·5	4570	266	229	4310	254	197	3710
272	259	4870	279·5	178	3345	298	47·5	843
281	182	3420	315·5	73·3	1377	—	—	—
311	81·4	1530	319	74·0	1391	—	—	—
317·5	74·4	1397	—	—	—	—	—	—
325	103	1938	—	—	—	—	—	—

selected for use. Table 2 indicates that the blank varies in different samples of blood, and it is therefore necessary to analyse a sample of normal blood with the unknowns, and to subtract the blank in the usual way. It is probable that the blank could be further reduced, for example, by introducing chromatographic techniques; but accurate analyses are readily possible without thus increasing the complexity of the method. Urine must be analysed by constructing full absorption spectra of extracts because only traces of β-naphthoxyethanol are excreted (Spinks, 1950).

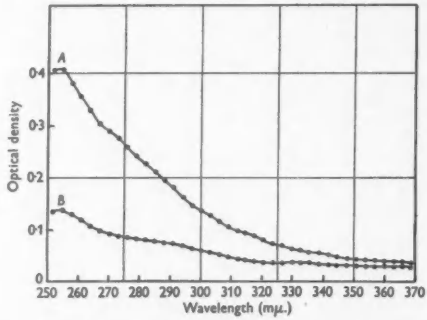


Fig. 4. Absorption spectra of chloroform extracts (40 ml.) of normal rabbit blood (*B*, 2 ml.) and urine (*A*, 2 ml.), buffered with Na_2HPO_4.

METHOD

Apparatus. Glass apparatus is washed in soap and alkali to remove adhering biological material, then in tap water, chromic-nitric acid mixture, tap water and distilled water in that order, and is dried in the oven. Just before use it is rinsed with $CHCl_3$. The most suitable spectrophotometer is a photoelectric type such as the Beckman, but a Hilger Spekker photometer and medium quartz spectrograph have been used successfully.

Reagents. 0·2 M-Na_2HPO_4; $CHCl_3$ (B.P.); 1% stock solution of β-naphthoxyethanol in ethanol (w/v); 5 mg./100 ml. standard solution of β-naphthoxyethanol. $CHCl_3$ (B.P.) contains ethanol as a preservative, but has been found to be satisfactory. The 5 mg./100 ml. standard solution of β-naphthoxyethanol is freshly prepared by diluting 0·5 ml. of the 1% stock solution to 100 ml. with distilled water.

Procedure. Pipette 2 ml. of blood into 4 ml. of 0·2 M-Na_2HPO_4 in a 60 ml. glass-stoppered bottle. Add 40 ml. of $CHCl_3$ and shake vigorously for 5 min. When the lower layer has separated, remove it by means of a pipette provided with a rubber teat and filter it into a 10 cm. silica spectrophotometer cell. Read the optical density (*a*) at 272 and 325 mμ. against a blank. The blank and a standard are prepared by substituting 2 ml. of water and 2 ml. of a 5 mg./100 ml. solution of β-naphthoxyethanol for blood in the above procedure. Normal blood (2 ml.) from the same animal is also analysed. The optical densities of the extracts of standard (*b*) and normal blood (*n*) are also read at 272 and 325 mμ. Then the concentration (*c* mg./100 ml.) of β-naphthoxyethanol in the unknown is given by the formulae:

$$c_{272} = 5(a_{272} - n_{272})/b_{272} \quad \text{and} \quad c_{325} = 5(a_{325} - n_{325})/b_{325}.$$

The two results should not differ by more than 10%. Their average is taken.

Table 2. *Effect on the apparent β-naphthoxyethanol concentration of normal blood and urine of extracting with chloroform at varying* pH

	Apparent concentrations of β-naphthoxyethanol (mg./100 ml.)									
	0·2 M-KH_2PO_4		0·2 M-Na_2HPO_4		10% Na_2CO_3		N-NaOH		2·5 N-NaOH	
Material	272 mμ.	325 mμ.	272 mμ.	325 mμ.	272 mμ.	325 mμ.	272 mμ.	325 mμ.	272 mμ.	325 mμ.
Rat blood	0·97	0·93	0·80	0·77	0·67	0·72	0·68	0·74	1·57	1·28
Rat blood	1·57	1·73	0·98	1·00	0·83	0·69	0·38	0·32	—	—
Rat blood	1·44	1·31	1·00	0·93	0·83	0·91	0·72	0·83	1·11	0·89
Rabbit blood	1·17	1·37	0·74	0·63	0·46	0·47	0·40	0·42	0·43	0·42
Rabbit urine	3·66	5·22	2·23	1·87	3·04	3·79	3·61	3·18	3·14	2·71

Table 3. *Recovery of β-naphthoxyethanol from blood* (2 *ml.*)

Added (μg.)	Found (μg.)	Recovery (%)
25	23·7	95
50	52·0	104
50	47·2	94
50	50·6	101
100	93·3	93
100	97	97
100	103	103
100	100	100
150	152	101
200	196	98
	Mean	99

Notes. An alternative method is available, using 10 ml. of benzene as solvent instead of 40 ml. of $CHCl_3$. The extracts are examined in 2 cm. cells. The blank in normal blood is thereby reduced almost to zero, but the optical density can be read only at 325 mμ., at which sensitivity is low. However, this method is useful when it is not possible to obtain a sample

Table 4. *Comparison of the concentrations of β-naphthoxyethanol found in the blood of dosed rabbits by determination at* 272 *and* 325 *mμ.*

Concentration found (mg./100 ml.)			
272 mμ.	325 mμ.	272 mμ.	325 mμ.
5·52	5·22	2·28	2·27
3·85	3·77	2·08	2·02
3·47	3·39	2·04	1·94
2·87	2·74	1·54	1·32
2·41	2·41	1·18	1·13
		0·81	0·70

of normal blood. The validity of the procedure using $CHCl_3$ has been established in three ways. (1) The recovery of β-naphthoxyethanol from blood (Table 3) and urine has been examined. (2) The specificity of the method has been checked by establishing the identity of the ratio d_{272}/d_{325} for β-naphthoxyethanol extracted from the blood of experimental

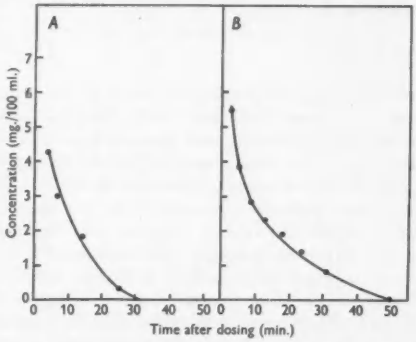


Fig. 5. Blood concentrations of β-naphthoxyethanol after intravenous administration of 80 mg./kg. to sheep (*A*) and rabbit (*B*).

animals, with that for authentic β-naphthoxyethanol (Table 4). (3) The method has been used for some time to determine β-naphthoxyethanol in the blood of sheep, horse and rabbit. Its accuracy is adequately illustrated by the smoothness of the typical curves shown in Fig. 5. The curves also illustrate the rapid elimination of β-naphthoxyethanol.

DISCUSSION

The development of photoelectric spectrophotometers (Beckman, Hilger, Unicam, etc.) has greatly facilitated the estimation of new drugs and related compounds, most of which contain aromatic or heterocyclic structures, and therefore show selective absorption in the ultraviolet. It is the practice in this laboratory, where ten to twenty new compounds are estimated annually, to examine a compound, first for fluorescence, and secondly for ultraviolet absorption. Only rarely has recourse to be had to alternative methods of estimation. The disadvantages of ultraviolet analysis are lack of specificity, and, very often, low sensitivity. These disadvantages can be partly neutralized by the proper choice of extraction conditions. The extraction of interfering materials from normal blood, and often of metabolites (Brodie, Udenfriend & Baer, 1947) is reduced by selecting a solvent of low polarity; moreover, the latter is less likely to give emulsions when shaken with large volumes of biological fluids. On the grounds of both specificity and sensitivity, therefore, the order of preference is, for example, light petroleum, benzene, chloroform, butanol. The least polar solvent that extracts the compound quantitatively is chosen. In the present study light petroleum did not extract the compound quantitatively, and chloroform was preferred to benzene because it permitted analysis at two wavelengths, a useful device that increases accuracy and confers an increased degree of specificity.

β-Naphthoxyethanol is eliminated very rapidly from the blood in conformity with its use as a short-acting anaesthetic. In the experiments illustrated here (Fig. 5), and other similar ones, it was possible to correlate the degree of anaesthesia with the blood concentration of β-naphthoxyethanol. For example, the reappearance of the righting reflex in the rabbit was usually associated with a concentration of about 2 mg./100 ml. The rapid elimination is effected by degradative processes, which are described in the following paper (Spinks, 1950).

SUMMARY

1. β-Naphthoxyethanol has been determined in blood by extracting it with chloroform and measuring the optical densities of the extract at the absorption maxima of 272 and 325 mμ.

2. β-Naphthoxyethanol is rapidly eliminated from the blood, in conformity with its use as a short-acting anaesthetic.

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Metabolism of β-Naphthoxyethanol in the Rabbit

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After the development of the method of determining β-naphthoxyethanol described in the preceding paper (Spinks, 1950) the excretion in rabbit urine was examined. Since normal urine contains chloroform-soluble constituents which absorb light of wavelengths 272 and 325 mμ. the technique adopted was to determine the normal daily excretion of such constituents in terms of milligrams of apparent β-naphthoxyethanol, and then to give the drug and continue the daily estimations. In no experiment was any significant increase in the daily excretion observed, and it was concluded that the drug is wholly metabolized in the rabbit. The absence from the urine of a compound which is completely cleared from the blood within 30–60 min. after intravenous administration (Spinks, 1950) was noteworthy, and it was decided to study its metabolism. Further support for this study was provided by a symptom first observed in the horse by the author's colleague, Mr Parry. About one horse in three anaesthetized with β-naphthoxyethanol excreted dark urine, appearing greenish brown by transmitted light and brown by reflected light. Further darkening usually occurred on standing. The absorption spectra of all the samples examined (cf. Fig. 1) were non-specific, and tests for pathological amounts of urobilinogen, haematoporphyrin, uroporphyrin, coproporphyrin and bilirubin were negative. It was, therefore, thought likely that β-naphthol had been formed from the drug, de-etherification being a well-known metabolic process (Williams, 1947). The administration of β-naphthol to dogs has been shown to result in darkening of the urine, ascribed to the formation *in vivo* of dihydroxynaphthalenes, which blacken rapidly in air (Lesnik & Nencki, 1886). If β-naphthol were a metabolite of the drug, it was obviously desirable to establish that it was not formed in large amount in view of its known toxicity. Since the difficulty of collecting total urine from the horse, and therefore of balancing metabolite excretion against drug intake, is great, it was decided to carry out the study in the rabbit. The administration of β-naphthoxyethanol to the rabbit leads to the excretion of brownish green urine, not usually, however, as dark as that from the horse.

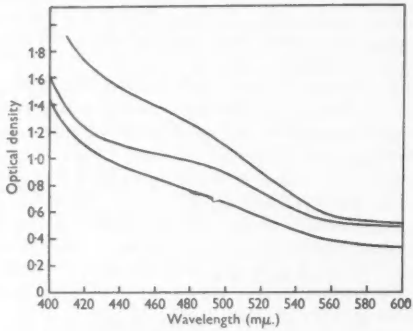


Fig. 1. Absorption spectra of dark brown urine samples from horses receiving β-naphthoxyethanol (1 in 10 dilutions, 2 cm. cells).

EXPERIMENTAL

It was thought that the most probable metabolites were β-naphthol and other phenols; conjugated derivatives of these and of β-naphthoxyethanol; quinonoid compounds; and, possibly, β-naphthoxyacetic acid. Since all of these should show characteristic ultraviolet absorption spectra, the method selected was to extract urine and 'hydrolysed' urine at weakly basic and at acid pH, and to construct the absorption spectra of the extracts. Each of the suggested metabolites should have been traceable under one of these four sets of conditions.

Methods

Animals. The rabbits were chinchilla bucks weighing 2–3 kg.

Dosage. β-Naphthoxyethanol was administered as a 10% (w/v) dispersion.

Absorption spectra. All absorption spectra were constructed using the Beckman quartz spectrophotometer, model DU. Readings were taken at 2 mμ. intervals in the ultraviolet, and at 4 mμ. intervals in the visible region; 2 cm. cells were used throughout.

Melting points. All melting points are uncorrected.

Spectrophotometric study

In a typical experiment two rabbits received a total of 1·7 g. of β-naphthoxyethanol intraperitoneally. The combined 24 hr. urine totalled 222 ml.

Free non-acidic metabolites. Urine (1 ml.) was treated with 1 ml. of 0·2M-Na_2HPO_4 and shaken for 5 min. with 40 ml. of

$CHCl_3$. The extract was separated, dried over Na_2SO_4 and examined in the spectrophotometer. The spectrum (*A*, Fig. 2) was non-characteristic and showed no evidence of the presence of any naphthalene derivative. In this fraction would have appeared β-naphthoxyethanol, solvent-soluble phenolic compounds and quinones of low molecular weight. All could therefore be present only in negligible amount.

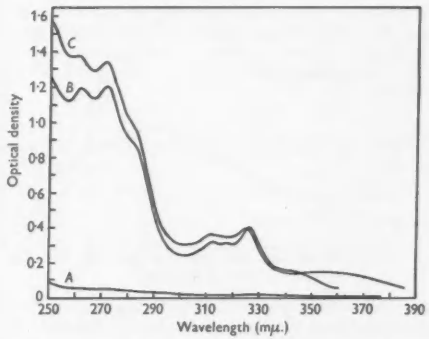


Fig. 2. Absorption spectra of: *A*, a chloroform extract of urine buffered to pH 8·5; *B*, a chloroform extract of urine acidified with HCl; *C*, a carbonate extract of *B*.

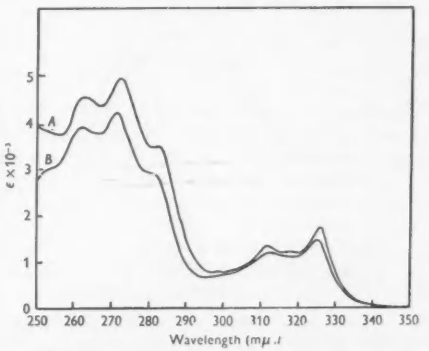


Fig. 3. Absorption spectra of authentic β-naphthoxyacetic acid: *A*, in chloroform ($\lambda_{max.}$ 262, 272, 282, 311, 318 and 325·5 mμ.; $\epsilon_{max.}$ 4575, 4940, 3550, 1390, 1252 and 1770 respectively); *B*, in 1% (w/v) Na_2CO_3 ($\lambda_{max.}$ 262, 271, 280·5, 311·5 and 325 mμ.; $\epsilon_{max.}$ 3880, 4220, 2930, 1200 and 1460 respectively).

Free acidic metabolites. Urine (0·5 ml.) was treated with 1 ml. of N-HCl and extracted with 40 ml. of $CHCl_3$ as described above. The absorption spectrum (*B*, Fig. 2) was very similar to that of β-naphthoxyethanol itself, suggesting the presence in the urine either of an acid of similar chromophoric structure, presumably β-naphthoxyacetic acid, or of a conjugated derivative of β-naphthoxyethanol, immediately hydrolysed on adding HCl. The former possibility was established by extracting fraction *B* with an equal volume of 1% Na_2CO_3 (w/v). The $CHCl_3$ residue was similar in spectrum to *A*, but the spectrum of the carbonate extract (*C*) clearly indicated the presence of an acid. By comparing the extinction coefficients from spectra *B* and *C* with those of authentic β-naphthoxyacetic acid (prepared by the method of Spica, 1886) in the corresponding solvents (Fig. 3) it was found that approximately 25% of the drug could be accounted for as β-naphthoxyacetic acid.

Conjugated non-acidic metabolites. Urine (5 ml.) was treated with 5 ml. of N-HCl and heated at 100° for 1·5 hr. On cooling, 5 ml. of N-NaOH were added and the volume was adjusted to 20 ml. with distilled water. The filtered solution (4 ml.), equivalent to 1 ml. of the original urine, was treated with 4 ml. of 0·2 M-Na_2HPO_4 and extracted with 40 ml. of $CHCl_3$. The absorption spectrum (*D*, Fig. 4) showed

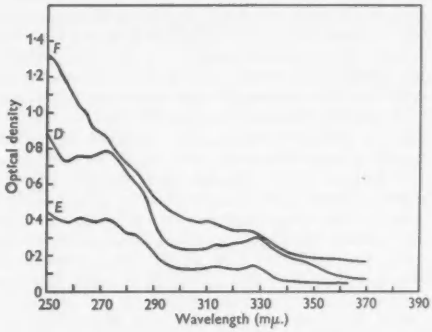


Fig. 4. Absorption spectra of: *D*, a chloroform extract of hydrolysed urine buffered to pH 8·5; *E*, the same as *D*, after washing with N-NaOH; *F*, a carbonate extract of acids from a chloroform extract of acidified, hydrolysed urine.

some evidence of maxima at the same wavelengths as β-naphthoxyethanol. The extract was purified by removal of phenols with N-NaOH. It then gave spectrum *E* (Fig. 4), which showed maxima at 263, 272, 314 and 327·5 mμ., and a marked inflexion at 282 mμ., suggesting that β-naphthoxyethanol was present. The amount was difficult to assess because the spectrum was clearly affected by other absorbing materials, but it did not seem that more than 1–2% of the drug could be accounted for in this fraction (see p. 184).

The spectrum of the phenolic fraction was inferred by subtracting *E* from *D*. It was clearly due to a complex mixture of compounds, and no further spectrophotometric study was made until isolation had been attempted (see p. 184).

Conjugated acid metabolites. The hydrolysed and neutralized urine (1 ml.), equivalent to 0·25 ml. of original urine, was acidified with 1 ml. of N-HCl, and extracted with 40 ml. of $CHCl_3$. The chloroform extract was shaken with an equal volume of 1% Na_2CO_3 (w/v), and the spectrum of the latter was constructed (*F*, Fig. 4). It was consistent with the presence in this fraction of the presumed β-naphthoxyacetic acid of the unhydrolysed urine, together with a mixture of other acids liberated by hydrolysis. The extinction coefficients did not suggest the formation by hydrolysis of further amounts of β-naphthoxyacetic acid.

Isolation experiments

The spectrophotometric study indicated the presence of β-naphthoxyacetic acid and conjugated β-naphthoxyethanol. These deductions were confirmed by isolation, and in addition β-naphthol was isolated as an azo derivative.

(I) *Isolation of β-naphthoxyacetic acid.* The main bulk of the urine (200 ml.) was acidified with an equal volume of N-HCl and extracted with 3 × 100 ml. of ether. The aqueous residue was retained for hydrolysis (see II below). The combined ether extracts were shaken with 3 × 30 ml. of 1% Na_2CO_3 (w/v) and the ether was discarded (cf. spectrum *A*). The combined carbonate extracts were acidified with conc. HCl. After standing overnight the dark, crystalline deposit was filtered, washed with water and dissolved in 25 ml. of boiling water. Charcoal (0·5 g., DY3) was added, boiling continued for 5 min. and the charcoal removed by filtration. The pale yellow filtrate was evaporated to incipient cloudiness and allowed to stand overnight. The colourless plates were filtered, washed with distilled water and dried *in vacuo* over $CaCl_2$. The yield was 130 mg., m.p. 151–153°; mixed with authentic β-naphthoxyacetic acid, m.p. 152–154°. One recrystallization from benzene gave rosettes (85 mg.), m.p. 154°. (Found C, 70·5; H 4·95. Calc. for $C_{12}H_{10}O_3$: C, 71·2; H, 4·95%.) The mother liquors were not further examined.

(II) *Isolation of β-naphthoxyethanol.* The acid urine residue from (I) was heated for 1·5 hr. at 100° and allowed to cool. It was then extracted with 3 × 150 ml. of ether. The urine was discarded, and the combined ether fraction washed with 2 × 50 ml. of water, and then extracted with 3 × 20 ml. of 1% Na_2CO_3. This extract did not give any crystalline product and was discarded. The ether residue was extracted with 3 × 20 ml. of 2N-NaOH and the combined aqueous fraction examined for phenolic metabolites (see III below). The ether fraction, now free from phenols and acids, was dried over Na_2SO_4 and the solvent removed on the steam bath. The tarry residue was dissolved in boiling light petroleum (b.p. 40–60°, 2 ml.) and the filtered solution allowed to stand overnight at room temperature. The precipitated solid weighed 8–10 mg. and melted at 62–66°. Recrystallization from light petroleum (b.p. 60–80°, 1 ml.) gave 6–7 mg. of almost pure β-naphthoxyethanol, m.p. 70–72°; mixed with an authentic specimen, m.p. 72–73°. The yield of crude material was equivalent to about 0·5% of the dose.

(III) *Isolation of β-naphthol as an azo derivative.* The NaOH fraction from (II) was acidified and extracted with 3 × 100 ml. of ether. The combined extracts were dried over Na_2SO_4, and the solvent was removed on the steam bath. The residue (20 mg.) was tarry and resisted attempts at crystallization. It was therefore decided to convert the phenols to azo dyes and to attempt a separation by chromatography. The residue was dissolved in 1 ml. of 2N-NaOH and treated with a solution of naphthalene-1-diazonium chloride (from 150 mg. of recrystalized α-naphthylamine hydrochloride). The mixture was acidified and shaken vigorously with 100 ml. of $CHCl_3$. The lower layer was separated, dried over Na_2SO_4 and passed through a column of activated alumina (7 × 1 cm.), the column being washed with 100 ml. of $CHCl_3$ to remove the last traces of dye (chromatogram 1). Some tarry impurities remained strongly adsorbed. The eluate gave absorption spectrum *G* of Fig. 5, which showed some similarity in position of the main maximum to that of 1-(naphthalene-1′-azo)-2-naphthol (prepared by the method of Meldola & Hanes, 1894; *K* of Fig. 6). The volume was reduced to 10 ml. and the solution rechromatographed on alumina (14 × 2 cm., chromatogram 2). A faint bluish band and a faint orange band at the top of the column were discarded. The remaining bands, a weakly adsorbed broad pink

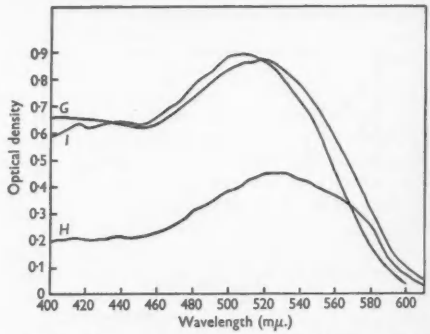


Fig. 5. Absorption spectra of chloroform solutions of mixed azo dyes from the conjugated phenolic fraction of the urine: *G*, total eluate from the first chromatogram; *H*, purple band from the second chromatogram; *I*, pink band from the second chromatogram.

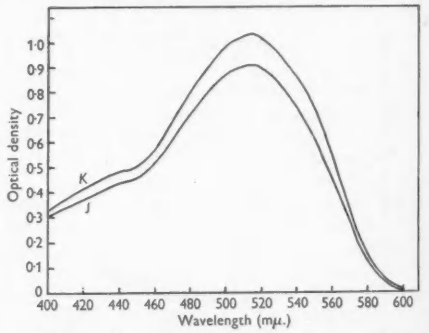


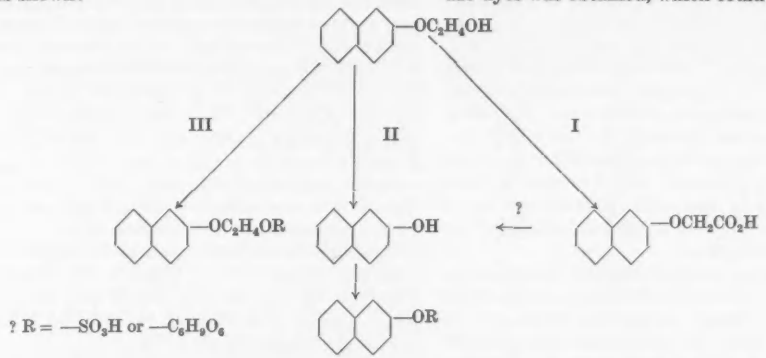
Fig. 6. Absorption spectra of 1-(naphthalene-1′-azo)-2-naphthol in chloroform: *K*, authentic sample ($\lambda_{max.}$ 512·5, $\epsilon_{max.}$ 17,600); *J*, sample from the conjugated phenolic fraction of urine.

band, and a more strongly adsorbed purple band were passed through the column by washing with 200 ml. of $CHCl_3$ and collected separately. The purple band gave spectrum *H* and the pink band spectrum *I* of Fig. 5. Both fractions were obviously mixtures, *I*, however, agreeing best with the spectrum of authentic 1-(naphthalene-1′-azo)-2-naphthol. This fraction was therefore evaporated to dryness, and the semi-crystalline residue dissolved in 10 ml. of benzene and chromatographed on alumina (14 × 2 cm.), the column being developed with benzene (chromatogram 3). Five main bands were observed as follows (in decreasing strength of adsorption): faint purple, purplish blue, orange, strong red, orange. The lower three bands were passed through the column by washing with 200 ml. of benzene, and collected separately. The red fraction was retained and the solvent removed to

give a crystalline residue (4·3 mg.). A trace dissolved in $CHCl_3$ gave a spectrum (*J* of Fig. 6) identical with that of authentic 1-(naphthalene-1′-azo)-2-naphthol. The remainder of the material was crystallized from ethanol $CHCl_3$ (70–30 (v/v), 0·5 ml.) to give needles (2·8 mg.) of 1-(naphthalene-1′-azo)-2-naphthol. (Found C, 80·3; H, 5·4. Calc. for $C_{20}H_{14}ON_2$: C, 80·4; H, 4·7 %); m.p. 227–228°, mixed with an authentic specimen, m.p. 227–228°. The yield was equivalent to about 0·1 % of the dose. The absence of β-naphthol from the free phenol fraction of the urine (and from the α-naphthylamine used) was readily demonstrated, no characteristic pink band being observed in the chromatograms.

DISCUSSION

β-Naphthoxyethanol is metabolized by the three routes shown:



Route I, to β-naphthoxyacetic acid, accounts for about 25 % of the drug (by spectrophotometric estimation); routes II and III account for about 0·5 and 0·1 % respectively (from amounts isolated). Each route represents a well-known metabolic reaction (Williams, 1947), and the main points of interest are the small amount of drug degraded by routes II and III, and the fact that about 75 % was not accounted for at all. Some of this may have been present as polyphenolic compounds (which could have remained in the aqueous phase under the conditions of extraction used) and their coloured oxidation products, but it is probable that much of the drug was either burned completely or converted to metabolites not specifically sought during these experiments, e.g. phthalic acid. The trace of β-naphthol isolated is of course negligible from the standpoint of toxicity. The most notable feature of the various degradative reactions, known and unknown, is their rapidity. Together they result in the reduction of blood concentrations of 5 mg. or more of the drug per 100 ml. to zero within half an hour to an hour.

The experiments were repeated using urine from horses receiving β-naphthoxyethanol. Techniques and results were so similar that a detailed description would be superfluous. A trace of free β-naphthoxyethanol was detected spectrophotometrically, and the presence of large amounts of β-naphthoxyacetic acid was confirmed by isolation. Its purification was more difficult than that of the material from rabbit urine, because of the presence of considerable quantities of other acids; only hippuric acid could be isolated from the mother liquors. Conjugated β-naphthoxyethanol was present, but in traces only, as in rabbit urine. The isolation of β-naphthol was attempted, but failed because a complex mixture of azo dyes was obtained, which could not be resolved satisfactorily by any chromatographic technique tried; normal horse urine gave a superficially similar mixture, but the presence of traces of β-naphthol in the experimental urine cannot be excluded.

SUMMARY

1. β-Naphthoxyethanol was not detected in the urine of rabbits receiving large doses intravenously or intraperitoneally.

2. Three metabolites have been identified as β-naphthoxyacetic acid (approx. 25 %), conjugated β-naphthoxyethanol (approx. 0·5 %) and conjugated β-naphthol (approx. 0·1 %).

3. It is suggested that part of the missing drug appears in the urine as coloured oxidation products of β-naphthol or other phenolic metabolites, but much must be degraded by unknown routes.

4. Traces of free and conjugated β-naphthoxyethanol and large amounts of β-naphthoxyacetic acid were detected in horse urine.

The author is indebted to Mr H. B. Parry for much helpful discussion.

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The Quantitative Determination and Identification of Pregnane-3α:20α-diol* in the Urine of the Pregnant Rabbit

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Although it has been shown by Heard, Bauld & Hoffman (1941), Hoffman & Browne (1942), Hoffman (1942) and Westphal (1942) that pregnanediol is present in the urine of rabbits following the administration of progesterone, the presence of pregnanediol of endogenous origin in the urine of pregnant rabbits has hitherto not been reported. In fact, Buxton & Westphal (1939) and Westphal (1942) examined the urine of pregnant rabbits for this end product of progesterone metabolism, but were unable to detect its presence. In the belief that previous failures to detect pregnanediol in the urine of rabbits during pregnancy might be due as much to shortcomings in the methods employed as to a low level of excretion, a reinvestigation of the problem was undertaken.

Recently, a sensitive method for the determination of pregnanediol in human urine has been described by Sommerville, Gough & Marrian (1948), and it seemed possible that this method might be applicable to rabbit urine. A series of preliminary experiments on the urine of male rabbits injected with progesterone and on acid-hydrolysed urine from male rabbits after the addition of pure pregnane-3α:20α-diol in varying amounts, showed that the method was, indeed, suitable for the purpose and that as little as 0·25 mg. of pregnanediol could be determined with a satisfactory degree of accuracy in one-half of a 24 hr. specimen of rabbit urine.

The application of this method to the urine of mated female rabbits yielded surprising results. In every instance in which the urine collected immediately after mating was examined, an excretion of 'pregnanediol' varying from about 0·5 to 2·8 mg./24 hr. and lasting for 1–3 days was observed. Following this transient post-copulatory excretion of 'pregnanediol', the values fell and remained at the 'blank' level of about 0·10–0·15 mg./24 hr. until the eight to tenth day, when the 'pregnanediol' rose again and was maintained at a level varying between 0·25 and 0·70 mg./24 hr. until shortly before parturition.

* *Editorial note.* The indices α and β for substituents of known orientation in steroids will in future be shown in the *Biochemical Journal* without parentheses. This practice, suggested by Fieser & Fieser (1949), has recently been adopted by the *Journal of the Chemical Society* and by *British Abstracts*.

Since the sulphuric acid colour reaction, by which pregnanediol is finally determined in this procedure, is not specific for this steroid, it could not be assumed at this stage of the work that the 'pregnanediol' excreted immediately after copulation or that excreted later in pregnancy was, in fact, pregnane-3α:20α-diol. Accordingly, attempts were made to isolate the sulphuric acid chromogen from the pooled extracts of urine specimens from a large number of rabbits. Urine was collected during the 24 hr. following mating and also during the last 20 days of pregnancy. From both batches of pooled extract pregnane-3α:20α-diol was isolated and identified as such unequivocally. Furthermore, the amounts isolated were of the same order of magnitude as those which were anticipated to be present on the basis of the previous colorimetric determinations. There can be little doubt therefore that most, if not all, of the 'pregnanediol' excreted at both periods is indeed pregnane-3α:20α-diol.

Experiments which throw some light on the origin of the pregnanediol excreted immediately after mating have been carried out by one of the present authors (W. G. V.) and will be published elsewhere.

EXPERIMENTAL

Determination of pregnanediol in rabbit urine

Collection of urine and application of the method of Sommerville et al. (1948). Rabbits fed on a pellet diet (Diet 43, Associated London Flour Millers Ltd.) and receiving 200–300 ml. of water daily were housed in metabolism cages, and the urine collected over 24 hr. periods in vessels containing 0·5 ml. toluene as a preservative. Catheterization was not employed and accordingly the specimens obtained did not represent 24 hr. collections exactly.

Since rabbit urine is usually alkaline, the specimens were made faintly acid to litmus with HCl in order that the pH should be similar to that of human urine. Each specimen was then filtered through glass wool, made up to 500 ml. with water, and divided into two 250 ml. samples for the duplicate determinations. Since this volume is half that employed in the original method, all volumes of reagents and solvents used in the determination were halved.

The only other modifications of the original method which were introduced were the following: (i) In the precipitation procedure for the purification of the pregnanediol the times of incubation at 37° were increased to 24 hr. in every case. (ii) The charcoal treatment for the removal of interfering

pigment was found to be unnecessary with rabbit urine. Accordingly, it was replaced by a simple filtration in ethanolic solution through a Whatman no. 1 paper. (Miss E. Sutherland has informed the authors that material giving a yellow colour with H_2SO_4 may sometimes be eluted from filter paper by ethanol. Accordingly, it is now considered to be essential that all filter papers used in the determination of pregnanediol by this method should be thoroughly washed with hot ethanol before use.)

Hydrolysis of the conjugated pregnanediol in rabbit urine. Since Hoffman (1942) has found that after the administration of progesterone to rabbits the pregnanediol in the urine is 'for the most part if not entirely' present as the glucuronide, it seemed probable that conditions of acid hydrolysis giving the maximum yield of free pregnanediol from human urine would also be optimal for rabbit urine containing conjugated pregnanediol. However, it could not be assumed that this was necessarily the case, and, accordingly, experimental verification was sought.

In a preliminary experiment several male rabbits were injected with progesterone and the urine collected during the following 48 hr. was pooled and diluted. Samples of the pooled urine were boiled for 5, 10, 15 and 20 min. after the addition of 0·1 vol. of conc. HCl and the pregnanediol determined in the usual manner. The results indicated that 10 min. boiling gives optimal hydrolysis of the conjugated pregnanediol as it does with human urine.

Later in the course of this work, after it had been demonstrated that pregnanediol of endogenous origin is present in the urine of pregnant rabbits, a similar experiment was carried out on pooled 24 hr. urine specimens obtained from four rabbits 13–15 days pregnant. The results, shown in Table 1, clearly support the conclusion arrived at from the preliminary experiment with the urine of progesterone-injected male rabbits.

Table 1. *Hydrolysis of conjugated pregnanediol in the urine of pregnant rabbits*

Time of boiling with 0·1 vol. of conc. HCl (min.)	Free pregnanediol found (mg./250 ml. diluted urine)
5	0·243
5	0·238
10	0·256
10	0·263
15	0·243
15	0·258
20	0·232
20	0·258

Recovery experiments. The sensitivity of the method and the accuracy of the procedures for extraction of the urine and purification of the pregnanediol were investigated in a series of recovery experiments in which pregnanediol was added to acid-hydrolysed urine from male rabbits. Three 24 hr. urine specimens were collected from three male rabbits, pooled, made up to 1500 ml. with water, and divided into six samples of 250 ml. Each sample was boiled under reflux for 10 min. after the addition of 25 ml. conc. HCl and then cooled. Two of the acid-treated samples were retained as 'blanks', while to the remaining four, pure pregnane-3α:20α-diol in ethanolic solution was added in two different known amounts in duplicate. This procedure was repeated on five occasions and the amounts of added pregnanediol were varied between 0·13 and 2·51 mg. The results of these experiments are shown in Table 2. It will be seen that whereas the recovery was unsatisfactory when 0·13 mg. was added to half a 24 hr. specimen, good recoveries resulted when 0·23 mg. or more was added.

Pregnanediol excretion of rabbits after mating and during pregnancy

The pregnanediol excretion was followed daily in three rabbits from 1 to 2 days before mating until 2 days after the birth of the litter (Figs. 1–3). In these three cases the transient excretion of pregnanediol immediately after mating and the prolonged excretion of the steroid during the latter two-thirds of pregnancy were well marked. In two rabbits (Figs. 4 and 5) urine collections were not commenced

Table 2. *Recovery of pregnanediol added to acid-hydrolysed male rabbit urine*

Pregnanediol added to equiv. of half of 24 hr. urine (mg.)	Apparent pregnanediol recovery (mg.)	Pregnanediol recovery corr. for 'blank' (mg.)	(%)
0·00	0·045	—	—
0·00	0·030	—	—
2·51	2·50	2·46	98·4
2·51	2·47	2·43	97·1
1·26	1·28	1·24	99·0
1·26	1·24	1·20	96·2
0·00	0·018	—	—
0·00	0·023	—	—
1·00	0·99	0·98	97·5
1·00	1·05	1·03	102·5
0·50	0·491	0·471	94·2
0·50	0·484	0·464	92·8
0·00	0·033	—	—
0·00	0·037	—	—
0·50	0·540	0·505	101·0
0·50	0·523	0·488	97·6
0·25	0·295	0·250	100·0
0·25	0·272	0·237	94·8
0·00	0·030	—	—
0·00	—	—	—
0·45	0·448	0·418	92·1
0·45	0·447	0·417	92·1
0·23	0·236	0·206	90·0
0·23	0·235	0·205	90·0
0·00	0·060	—	—
0·00	0·037	—	—
0·25	0·266	0·218	87·2
0·25	0·275	0·222	88·8
0·13	0·120	0·072	57·6
0·13	0·118	0·070	56·0

until 24 hr. after mating, and in these cases the transient post-copulatory excretion of pregnanediol was missed. In order to confirm the transient excretion of pregnanediol after mating, the pregnanediol excretion levels of two rabbits were followed from the day before mating until a few days after (Figs. 6 and 7).

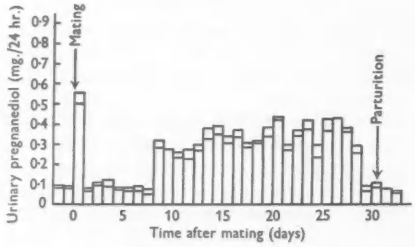

Fig. 1. Pregnanediol excretion of pregnant rabbit.

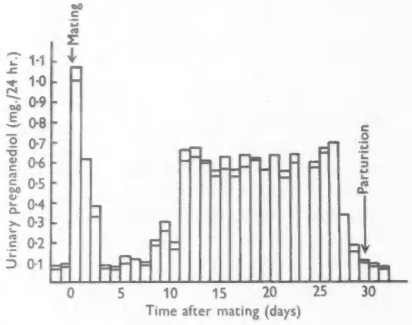

Fig. 2. Pregnanediol excretion of pregnant rabbit.

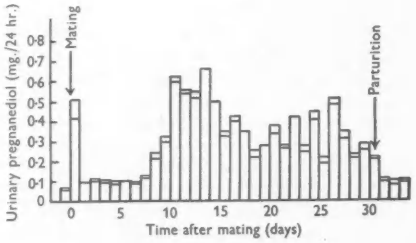

Fig. 3. Pregnanediol excretion of pregnant rabbit.

In these Figures the values for the duplicate determinations are shown by the double lines at the tops of the columns. It should be noted that values of less than 0·50 mg./24 hr. have no strict quantitative significance.

Isolation of pregnane-3α: 20α-diol from the urine of pregnant rabbits (twelfth day until term)

Complete 24 hr. urine samples were collected with toluene as preservative from five pregnant Chinchilla

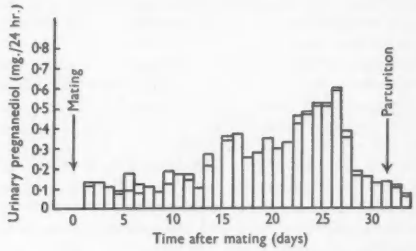

Fig. 4. Pregnanediol excretion of pregnant rabbit.

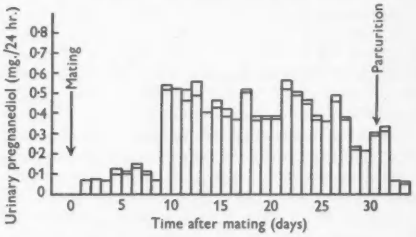

Fig. 5. Pregnanediol excretion of pregnant rabbit.

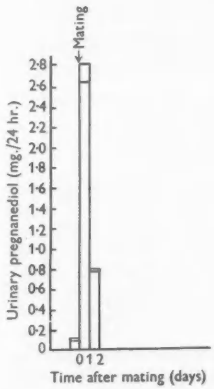

Fig. 6.

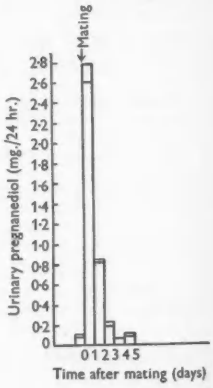

Fig 7.

Fig. 6. Pregnanediol excretion of rabbit (female) after mating.

Fig. 7. Pregnanediol excretion of rabbit (female) after mating.

rabbits from the twelfth day after mating until term. The samples were stored in the refrigerator and worked up at intervals of 3 or 4 days in the following

manner: three or four successive samples from each rabbit were pooled and diluted to 1000 ml. Each diluted, pooled sample was made just acid to litmus with HCl and heated to boiling under reflux with 200 ml. of toluene. Concentrated HCl (100 ml.) was then added and the boiling continued for 10 min. After cooling, the mixture was transferred to a separating funnel, shaken, and the aqueous layer run off from the upper toluene-emulsion layer. The aqueous layer was re-extracted twice more with 200 ml. vol. of toluene and the combined toluene emulsions filtered through a Büchner funnel with gentle suction. The filtrate was then transferred to a separating funnel and the aqueous phase run off. The toluene extract, after washing with 2 × 200 ml. of N-NaOH and 2 × 200 ml. of water, was evaporated to a small volume on a hot plate and then to dryness under reduced pressure in a boiling water bath. The 'toluene-soluble neutral fractions' so obtained were stored in a vacuum desiccator until the completion of the working up of all the urine samples.

The 'toluene-soluble neutral fractions' were combined, and after treatment in the usual manner with trimethylammoniumacetohydrazide chloride (Girard & Sandulesco, 1936) yielded 0·739 g. of non-ketonic material.

An attempt to purify the pregnanediol assumed to be in this material by the Astwood & Jones (1941) precipitation procedure was unsuccessful, since after two successive precipitations from ethanolic solution by 0·1 N-NaOH and water respectively, only 4·9 mg. of solid material were obtained. Accordingly, the combined filtrates from these precipitations, after removal of most of the ethanol by distillation under reduced pressure, were extracted with ether, and the extract evaporated to dryness after washing with water. The non-ketonic material recovered in this manner weighed only 0·309 g.

This material, dissolved in 20 ml. of benzene, was poured on to a column of 9 g. of Al_2O_3 (Peter Spence, Type H; dried at 100° *in vacuo*) and the column eluted successively with eight 20 ml. portions of benzene, twelve 20 ml. portions of anhydrous ether, and seven 20 ml. portions of anhydrous acetone. The residues obtained by evaporation of the second and third acetone eluates contained considerable amounts of white crystalline material, and this was separated from the accompanying gum by chilling overnight in a small volume of acetone. A further small quantity of crystalline material was obtained in a similar manner from the first acetone eluate.

An attempt was made to purify this material by sublimation at 130°/10^{-5} mm. The product (23·6 mg.) was, however, grossly impure. Purification was effected by warming for a few minutes with 2 ml. of benzene, allowing to stand at room temperature for 2 hr. and then filtering off the solid. The benzene-insoluble solid so obtained weighed 17·7 mg. and melted at 232–237° (corr.). Crystallization of this from 1·5 ml. of methanol yielded 7·5 mg. of fine white needles, m.p. 234–237° (corr.). Mixed with authentic pregnane-3α:20α-diol (m.p. 237–238·5°, corr.), the melting point was 233–236° (corr.). Analysis of material dried at 80° for 2 hr. *in vacuo* over P_2O_5: C, 78·3; H, 11·1. Calculated for $C_{21}H_{36}O_2$: C, 78·8; H, 11·2%.

A further quantity of material (m.p. 233–236°, corr.) was obtained from the methanolic mother liquor after evaporation to dryness by two successive treatments with benzene in the manner described above. This was acetylated in the usual manner with acetic anhydride and pyridine, and the product crystallized from hexane. Well-formed stout needles melting at 162–163° and 176–179° (corr.) were obtained. Mixed with authentic pregnane-3α:20α-diol diacetate (m.p. 165–166° and 180–181°) the melting points were 162–163° and 176–179° (corr.).

It has been suggested that the double melting point of pregnane-3α:20α-diol diacetate, which was first observed by Marrian (1929), is characteristic of impure samples (Heard, Hoffman & Mack, 1944). In the experience of the present authors, this is not the case, since the diacetate, even after repeated recrystallizations from methanol and from hexane, still exhibits this phenomenon. Recrystallized samples may melt completely at the lower temperature, or after slight softening at the lower temperature may not melt completely until the higher temperature is reached. Samples melting at the lower temperature, if held at this temperature for a few minutes before melting is quite complete, may resolidify and then do not melt below the higher temperature. Melted samples which do not resolidify before they have supercooled below the lower melting point invariably melt completely at the lower temperature on reheating.

Isolation of pregnane-3α:20 α-diol from the urine of female rabbits after mating

Urine was collected from twenty-one female rabbits during the 24 hr. period immediately following mating. According to the number of specimens obtained on any 1 day, batches of two to five were pooled for hydrolysis and extraction with toluene. In order to avoid the difficulties encountered in the previously described isolation of pregnanediol from rabbit urine (see preceding section), the toluene-soluble fractions from each batch were purified immediately by the precipitation technique as described by Somerville *et al.* (1948). It was evident that the previous failure of the precipitation technique must have been due to the prolonged storage of the neutral fraction, or to the subsequently used Girard separation, or to both, since in the present experiment each batch of neutral fraction yielded light coloured solid material in about the expected amount.

On combination of the solid products obtained from the different batches, 21·8 mg. of slightly pigmented solid contaminated with traces of gum

were obtained. Some of the pigment and most of the gum was removed by leaching the solid first with about 1 ml. of ice-cold acetone and then with about 1 ml. of benzene. The material, after treatment with charcoal in boiling ethanol to remove the remaining pigment and crystallization from 1·25 ml. of methanol, yielded 7·8 mg. of fine white needles, m.p. 232–237° (corr.). Mixed with authentic pregnane-3α:20α-diol (m.p. 237–238·5°, corr.) the melting point was 233–237° (corr.).

Acetylation with acetic anhydride and pyridine yielded a product which, after treatment with charcoal in boiling *n*-hexane followed by crystallization from *n*-hexane, was in the form of stout needles, m.p. 163–165° and 179–181° (corr.). Mixed with authentic pregnane-3α:20α-diol diacetate (m.p. 165–166° and 180–181°, corr.) the melting point was 162–163° and 178–181° (corr.).

SUMMARY

1. It has been shown that, using the method of Sommerville *et al.* (1948), as little as about 0·25 mg. of pregnanediol can be determined with a satisfactory degree of accuracy in one-half of a 24 hr. specimen of rabbit urine.

2. By this procedure the excretion of 'pregnanediol' in the urine of pregnant rabbits has been detected and studied quantitatively. Immediately after mating a transient excretion of 'pregnanediol' lasting for 1–3 days occurs. This is followed at about the eighth to tenth day of pregnancy by a prolonged excretion of 'pregnanediol' which is maintained until just before parturition.

3. The 'pregnanediol' excreted immediately after mating and that excreted later in pregnancy in the rabbit have been isolated and identified as pregnane-3α:20α-diol.

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Lysine Analogues as Inhibitors of Bacterial Growth

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Many investigations during recent years have demonstrated that analogues of essential metabolites may exert antibacterial action, but this approach has not so far led to the synthesis of a practical chemotherapeutic agent. It seemed to us that the ubiquity of the amino-acids, coupled with their importance in the biosynthesis of enzymes and other proteins, warranted further investigation of amino-acid analogues and peptides as potential chemotherapeutic agents. As it was impracticable to prepare analogues of every amino-acid, the choice between various alternatives was guided by existing knowledge of amino-acid metabolism.

The majority of amino-acids can undergo rapid deamination and reamination at the α-amino group (Braunstein & Kritzmann, 1937; Schoenheimer, Ratner & Rittenberg, 1939). It seemed undesirable that a potential antimetabolite should be subject to rapid metabolic transformation, and we were, accordingly, faced with the problem of designing a series of amino-acid analogues of such metabolic stability that they might retain the characteristic α-amino-carboxylic acid grouping, during protein synthesis. Experiments with ^{15}N in rats have indicated that lysine was an exception in the general

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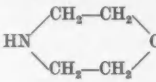
metabolic turnover, and did not receive either of its nitrogen atoms from the nitrogen of other amino-acids or from inorganic nitrogen (Schoenheimer *et al.* 1939). The failure of rats on a lysine-free diet to utilize the α-hydroxy analogue of lysine (McGinty, Lewis & Marvel, 1924) also supports the view that lysine does not participate in transamination reactions in the same way as the majority of amino-acids. This unique metabolic behaviour of lysine suggested that analogues of lysine might possess greater potentialities as antimetabolites than analogues of other amino-acids.

We accordingly synthesized and tested three types of lysine (I, $n=4$) analogue: (1) a higher straight chain homologue (I, $n=5$), 1:6-diaminohexane-1-carboxylic acid; (2) a C–N substituted analogue of lysine (II) in which the terminal amino group and the β-carbon atom of lysine were incorporated into a piperidine ring structure, β-4-piperidyl alanine; and (3) analogues formed by incorporating (II) in amide (peptide) linkage with ammonia or amino-acids.

$$NH_2.(CH_2)_n.CH(NH_2).COOH \qquad (I)$$



(II)

Synthetic methods

Several interesting points arose in the synthesis of the compounds listed in Table 1, and some unexpected difficulties were encountered in attempting to extend the range of compounds under test. Synthesis of 1:6-diaminohexane-1-carboxylic acid was accomplished by an extension of the acetamidomalonic ester synthesis described by Albertson & Archer (1945). Condensation of ethyl acetamidomalonate and 1-chloro-5-benzamidopentane was slow and the isolation of a crystalline salt of homolysine was extremely tedious.

Suitable intermediates for the synthesis of piperidyl-alanines were obtained by condensing α- and γ-picolines with chloral. Whereas 2-(3′:3′:3′-trichloro-2′-hydroxypropyl)-pyridine hydrochloride had previously been obtained in good yield (67 %; Tullock & McElvain, 1939), no satisfactory method existed for the synthesis of the corresponding 4-compound (cf. Alberts & Bachman, 1935). By keeping a mixture of γ-picoline and chloral at 37° for 8 days it was found that 4-(3′:3′:3′-trichloro-2′-hydroxypropyl)pyridine could be obtained in 68 % yield; the best yield previously reported for this compound was 16–18 % (Alberts & Bachman, 1935). Conversion of the trichlorohydroxypropylpyridines to the corresponding pyridylacrylic acids was achieved by the standard method.

Considerable difficulty seems to have been encountered by earlier investigators in attempts to reduce pyridylacrylic acids to the corresponding piperidylpropionic acids; for example, Rabe & Kindler (1919) reduced 4-pyridylacrylic acid by the use of sodium in amyl alcohol. No yields were quoted, but repetition of their method gave disappointing results. Tullock & McElvain (1939) report the failure of Raney nickel under high pressure, a failure which we can also confirm. It was found, however, that 2- and 4-pyridylacrylic acids could be quantitatively reduced at room temperature and atmospheric pressure by use of platinum oxide in the presence of a small excess of hydrochloric acid. In the absence of acid the aromatic ring remains completely unreduced, and in the presence of exactly one equivalent of acid, reduction is slow and incomplete (cf. Rubstov, 1946). The difficulty of catalytic reduction, as was pointed out by Maxted (1948), is probably associated with the existence on the pyridine nitrogen of a free electron pair which leads to the strong adsorption of such a nitrogen atom by a metal catalyst; this self-poisoning effect is eliminated in the presence of hydrochloric acid.

α-Halogenation of *N-benzoyl-2-(4-piperidyl)propionic acid* on a small scale by the method of Eck & Marvel (1934) gave no difficulty, and *N-benzoyl-2-(4′-piperidyl)-1-bromopropionic acid* was isolated in 75 % yield; on a larger scale, however, the reaction with phosphorus and bromine became violently exothermic and in the absence of a suitable solvent the reaction mixture became too viscous to allow effective stirring. The result was incomplete bromination and partial cleavage of the benzamide linkage. α-Chlorination by the method of Galat (1947) using sulphuryl chloride did not give such a good yield as the small-scale bromination with phosphorus and bromine (60 % compared with 75 %), but the reaction was mild and non-exothermic and was accordingly preferred for large-scale work.

Having successfully halogenated benzoyl-2-(4′-piperidyl)propionic acid no difficulty was anticipated in the halogenation of *benzoyl-2-(2′-piperidyl)propionic acid*; nevertheless, we were unable to find any conditions under which the 2-isomer could be α-halogenated without disruption of the *N*-benzoylpiperidyl radical. The corresponding *2-(p-toluenesulphonyl-2′-piperidyl)propionic acid* was prepared in the hope that the toluenesulphonyl-piperidyl group would be more stable than benzoylpiperidyl, but, under the conditions which had been successful with the 4-isomer, this compound was also degraded and toluenesulphonyl chloride was recovered in almost quantitative yield from the reaction mixture. It will be remembered that von Braun (1904) prepared 1:5-dichloropentane by reaction of benzoylpiperidine with phosphorus pentachloride. It seems probable that some similar reaction may take place with benzoyl-2-piperidylpropionic acid in the presence of bromine and phosphorus.

Conversion of *benzoyl*-2-(4′-*piperidyl*)-1-*chloropropionic* acid to 2-(4′*piperidyl*)-1-*aminopropionic acid* (piperidylalanine) was effected without difficulty. Dicarbobenzyloxypiperidyl-alanine was coupled with leucine methyl ester by the azide method of Bergmann & Zervas (1932). A curious and unexplained abnormality was encountered in this coupling; a considerable quantity of *dicarbobenzyloxy*-2-(4′-*piperidyl*)-1-*aminopropionamide* was formed as a by-product in the conversion of the acid hydrazide to the peptide through the acid azide. The formation of dicarbobenzyloxylysyl amide under similar conditions and in similar yield was reported by Prelog & Wieland (1946), but repeated experiments with other carbobenzyloxy-amino-acid azides did not lead to similar amide formation.

In coupling piperidylalanine with *p*-aminobenzoic acid the acid chloride method was found to be preferable to the azide method; the low basicity of the aromatic amino group of methyl *p*-aminobenzoate prevented rapid reaction between it and the acid azide of dicarbobenzyloxypiperidylalanine.

EXPERIMENTAL

1:6-*Diaminohexane*-1-*carboxylic acid*. To a hot solution of Na (2·3 g.) in dry ethanol (75 ml.) was added ethylacetamidomalonate (31·2 g.) followed by 1-chloro-5-benzamidopentane (17·45 g.). The mixture was boiled for 18 hr., the ethanol removed under reduced pressure, and the oily residue mixed with water. The product was extracted into ether, dried, and the ether removed. The residual oil (24·7 g.) was mixed with aqueous KOH (20%; 10 g.) and heated at 95° for 2 hr.; a water-insoluble oil was removed and the aqueous alkaline solution acidified with excess of HCl; an oil separated which could not be crystallized. Decarboxylation of the substituted malonic acid took place rapidly when the oil was boiled with 6N-HCl and after 10 hr., hydrolysis of the benzamide and acetamido groups was also complete. Benzoic acid was removed from the acid solution which was then concentrated to a thick syrup. The syrup was extracted with ethanol, the insoluble residue discarded and the ethanolic solution of the dihydrochloride concentrated slowly. 1:6-Diaminohexane-1-carboxylic acid could not be crystallized either as the dihydrochloride or as the dipicrate. The diamine-dihydrochloride was mixed with N-HCl, and phosphotungstic acid in N-HCl was added in excess. The solid phosphotungstate was collected, suspended in water and decomposed with baryta. The filtrate and washings from the Ba phosphotungstate were concentrated and left a colourless syrup (4·2 g.). The syrup was dissolved in the minimum volume of water and HCl added to bring the pH to 5·0. 1:6-*Diaminohexane*-1-*carboxylic acid monohydrochloride* crystallized from the solution after cautious addition of ethanol. The product was recrystallized from aqueous ethanol as transparent needles of the monohydrate, m.p. 176° (decomp.). (Found: C, 39·1; H, 8·9; N, 13·1. $C_7H_{16}O_2N_2$.HCl.H_2O requires C, 39·17; H, 8·86; N, 13·05%. Loss in weight at 105°/20 mm., 7·9. $1H_2O$ requires 8·4%.)

4-(3′:3′:3′-*Trichloro*-2′-*hydroxypropyl*)*pyridine* (I). Pure chloral (190 g.; b.p. 97°/755 mm.) and γ-picoline (106 g.) were mixed and kept at 37° for 8 days. The reaction product, a dark brown viscous oil, was diluted with ethanol (500 ml.) and heated three times with successive lots (20 g.) of charcoal. Concentration of the combined filtrates and washings left a light brown solid which crystallized from 50% aqueous methanol as needles, m.p. 160° (210 g.; 68% of theory; cf. Rabe & Kindler, 1919; Alberts & Bachman, 1935).

2-(4′-*Pyridyl*)*acrylic acid* (II). A solution of KOH (67·5 g.) in ethanol (350 ml.) was cooled in ice and an ethanolic solution of (I) (48 g.) was added slowly. The reaction mixture was stirred and the temperature allowed to rise slowly. The mixture was finally heated for 15 min. at 95°, cooled and the precipitated KCl removed. The brown residue remaining after evaporation of the ethanol was dissolved in water and neutralized with acetic acid. The sparingly soluble 2-(4′-pyridyl)acrylic acid (14·5 g.) separated slowly and was collected, m.p. 296°; the hydrochloride crystallized from ethanol as needles, m.p. 244° (cf. Rabe & Kindler, 1919).

2-(4′-*Piperidyl*)*propionic acid* (III). The hydrochloride of (II) (46 g.) in water (300 ml.) containing one equivalent of HCl was hydrogenated at room temperature and atmospheric pressure using Adams's platinum oxide catalyst. Hydrogenation was complete after 60 hr. The catalyst was removed and the solution concentrated. The product was crystallized from hot ethanol. During this procedure some esterification of the piperidylpropionic acid took place and only the first crop (30 g.) of crystalline material which separated on addition of acetone to the ethanolic solution was the hydrochloride of (III), needles, m.p. 244°. (Found: C, 49·8; H, 8·4; N, 7·3. $C_8H_{15}O_2N$.HCl requires C, 49·6; H, 8·3; N, 7·2%.) Addition of ether to the mother liquor of the acid hydrochloride gave a second crop of crystalline material (11 g.), m.p. 129–130°, which was identified as the hydrochloride of *ethyl*-2-(4′-*piperidyl*)*propionate*. (Found: C, 54·4; H, 9·3; N, 6·2. $C_{10}H_{19}O_2N$.HCl requires C, 54·2; H, 9·0; N, 6·3%.)

Benzoyl 2-(4′-*piperidyl*)*propionic acid* (IV). Benzoylation was carried out at 0° by dropwise addition of benzoyl chloride (15 g.) to an alkaline solution (50 ml. 2N-NaOH) of the hydrochloride of (III) (19 g.). A further equivalent of alkali (25 ml., 2N) was added during the period of benzoylation. The product separated on acidification of the reaction mixture and was crystallized from ethanol as white prisms, m.p. 148° (21·8 g.). (Found: C, 69·2; H, 7·4; N, 5·4. $C_{15}H_{19}O_3$ requires C, 69·0; H, 7·3; N, 5·4%.)

Benzoyl 2-(4′-*piperpidyl*)-1-*bromopropionic acid* (V). The method of bromination was essentially that of Eck & Marvel (1934). Benzoyl-2-(4′-piperidyl)propionic acid (4·9 g.) was mixed with red P (0·83 g.) and allowed to react at 0° with Br_2 (12 g.). The reaction was completed by heating and the α-bromo acid (5·0 g., 78% theory) was isolated by the method recommended by Eck & Marvel. After repeated crystallization from ethanol the product (3·4 g.) was obtained in the form of white plates melting at 183–184°. (Found: C, 53·3; H, 5·5; Br, 23·2. $C_{15}H_{18}O_3NBr$ requires C, 53·0; H, 5·3; Br, 23·5%.)

Benzoyl 2-(4′-*piperidyl*)-1-*chloropropionic acid* (VI). Chlorination was effected by the method of Galat (1947). Benzoyl 2-(4′-piperidyl) propionic acid (30 g.) was intimately mixed with powdered I_2 (0·5 g.) and the mixture added to SO_2Cl_2 (70 ml.). The reaction mixture was heated at 60–65° for 2 hr. and then gradually brought to the boil to complete the reaction. Sulphuryl chloride was removed under reduced pressure and the viscous residue poured into a mixture of ice and water. The material which separated gradually solidified.

The solid (33·5 g.) was ground to a fine powder, washed thoroughly, dried and dissolved in hot ethanol. The product crystallized from ethanol as pale yellow prisms (16 g.), m.p. 170°. The mother liquors were reworked by evaporation to dryness, trituration with benzene and crystallization of the benzene-insoluble residue from 80% ethanol. The combined crops of crystalline material (24 g.) were recrystallized from ethanol, yield 20 g. (60% theory), m.p. 174°. (Found: C, 60·6; H, 6·1; Cl, 12·4. $C_{15}H_{18}O_3NCl$ requires C, 60·9; H, 6·1; Cl, 12·0%.)

2-(*Benzoyl-4′-piperidyl*)-1-*aminopropionic acid* (VII). The chloroacid (VI) (11 g.) was mixed with NH_3 (175 ml., *d* 0·88) and heated under pressure for 12 hr. at 100°. Excess of ammonia was removed under reduced pressure and Cl^- eliminated by addition of Ag_2SO_4. Excess Ag was removed as the sulphide and SO_4 as $BaSO_4$. The product was a crystalline solid (7·5 g.), m.p. 216–218°, which was used for the next stage without further purification.

2-(*4′-piperidyl*)-1-*aminopropionic acid* (VIII). The product of the previous experiment (6·5 g.) was debenzoylated by boiling for 10 hr. with 6 N-HCl (120 ml.). The acid solution was freed from benzoic acid and concentrated to dryness. The residue was crystallized by the careful addition of ether to an ethanolic solution of the dihydrochloride containing a few drops of water. The pure dihydrochloride monohydrate (4·2 g.) separated from aqueous acetone or from a mixture of ethanol and ether as plates, m.p. 160°. (Found: C, 36·5; H, 7·6; N, 10·4. $C_8H_{16}O_2N_2.2HCl.H_2O$ requires C, 36·5; H, 7·6; N, 10·6%. Loss of weight at 120°, 6·6%. $1H_2O$ requires 6·8%. Anhydrous dihydrochloride: N, 11·6. $C_8H_{16}O_2N_2.2HCl$ requires N, 11·4%.)

Dicarbobenzyloxy-2-(*4′-piperidyl*)-1-*aminopropionic acid* (IX). The dicarbobenzyloxy derivative was prepared according to the method of Bergmann, Zervas & Ross (1935). From 4 g. of 2-(4′-piperidyl)-1-aminopropionic acid dihydrochloride we obtained 6·2 g. of the dicarbobenzyloxy derivative as a colourless syrup which could not be crystallized.

Dicarbobenzyloxy-2-(*4′-piperidyl*)-1-*aminopropionamide* (X). The product (IX) from the above experiment was esterified by treatment with excess diazomethane. A portion of the ester (1·8 g.) was mixed with excess ammoniacal methanol (saturated at 0°) and heated under pressure at 37° for 48 hr. The solvent was removed and the product crystallized from ethanol, prisms, m.p. 134° (1·4 g.). (Found: C, 65·4; H, 6·5; N, 9·8. $C_{24}H_{29}O_5N_3$ requires C, 65·6; H, 6·6; N, 9·6%.)

2-(*4′-Piperidyl*)-1-*aminopropionamide dihydrochloride* (XI). Compound (X) (1·1 g.) in methanol (30 ml.) containing 2 equiv. acid (0·18 ml. of 10 N-HCl) was hydrogenated in the presence of palladium black (300 mg.). When evolution of CO_2 was complete (3 hr.) the catalyst was removed, the solvent evaporated, and the residual amide dihydrochloride (0·5 g.) crystallized as plates from a mixture of methanol and acetone, m.p. 292° (decomp.). (Found: C, 39·1; H, 7·9; N, 17·5. $C_8H_{17}ON_3.2HCl$ requires C, 39·3; H, 7·8; N, 17·2%.)

Dicarbobenzyloxy - 2 - (*4′* - *piperidyl*) - 1 - *aminopropionic acid hydrazide* (XII). Excess of hydrazine (1 ml., 90%) was added to a solution of dicarbobenzyloxy 2-(4′-piperidyl)-1-aminopropionic acid methyl ester (2 g.) in methanol (20 ml.). After 24 hr. at room temperature the solvent was removed from the mixture and the residual oil crystallized from a mixture of ethyl acetate and ligroin. The pure hydrazide (1·8 g.) separated as plates, m.p. 115°. (Found: C, 63·7; H, 6·6; N, 12·3. $C_{24}H_{30}O_5N_4$ requires C, 63·4; H, 6·6; N, 12·3%.)

Dicarbobenzyloxy - 2 - (*4′* - *piperidyl*) - 1 - *aminopropionyl*-DL-*leucine methyl ester* (XIII). A. *Azide method.* The hydrazide (XII) (1·6 g.) was dissolved in glacial acetic acid (5 ml.) and diluted with N-HCl (15 ml.). The mixture was cooled to 0° and a cooled solution of $NaNO_2$ (1·1 g.) in water (10 ml.) was added dropwise to the stirred solution. The precipitated acid azide was extracted into ethyl acetate (50 ml.) and the ethyl acetate washed successively at 0° with pre-cooled water, with cooled saturated $NaHCO_3$ solution and again with water. The neutral solution of azide in ethyl acetate was quickly dried over Na_2SO_4 and added during 10 min. to a cooled solution of leucine methyl ester (0·5 g.) in ether (25 ml.). The mixture was left for 24 hr. and then extracted with N-HCl. Excess leucine ester was recovered from the aqueous acid solution. The organic solvent mixture was then washed with $NaHCO_3$, with water, and dried over Na_2SO_4. Removal of solvent *in vacuo* left a pale yellow gum (0·6 g.) which crystallized after trituration with ethyl acetate. The crystalline product (0·2 g.) was not the dipeptide but dicarbobenzyloxy-2-(4′-piperidyl)-1-aminopropionamide; plates, m.p. 132–133°. (Found: C, 65·9; H 6·6; N, 9·7. $C_{24}H_{29}O_5N_3$ requires C, 65·6; H, 6·6; N, 9·6%.) The required dipeptide, m.p. 104° (0·25 g.), was isolated from the ethyl acetate mother liquors of the amide by addition of light petroleum. (Found: C, 65·8; H, 7·4; N, 7·6. $C_{31}H_{41}O_7N_3$ requires C, 65·6; H, 7·2; N, 7·4%.)

B. *Acid chloride method.* As an alternative to the above method the same peptide was prepared by the conversion of the acid (IX) (1·0 g.) to the acid chloride by treatment with PCl_5 (0·5 g.) in ether (20 ml.) at 0°. When the PCl_5 had entirely dissolved, the solution was filtered and diluted with light petroleum. The solvent was decanted from the precipitated acid chloride which was immediately dissolved in ether, washed quickly in ice-cold water (10 ml.) and dried over Na_2SO_4. DL-Leucine methyl ester hydrochloride (1·5 g.) was dissolved in water (10 ml.) and the aqueous solution covered with ether (20 ml.). The mixture was cooled to −10° and the amino-acid ester forced into the ether layer by gradual addition of a large excess of anhydrous K_2CO_3. The ether layer was decanted from the K_2CO_3 sludge, quickly dried over Na_2SO_4 and filtered. The acid chloride solution already prepared was added dropwise, with shaking, to 2 equiv. of the ester solution, and after 1 hr. the leucine methyl ester hydrochloride which had separated was removed by filtration. The ethereal filtrate was washed successively with water, N-HCl, $KHCO_3$ (7%) and water, dried and concentrated *in vacuo*. The product crystallized from a mixture of ethyl acetate and light petroleum as platelets, m.p. 104–106° (0·25 g.) (Found: C, 65·9; H, 7·4; N, 7·8. $C_{31}H_{41}O_7N_3$ requires C, 65·6; H, 7·2; N, 7·4%.)

2-(*4′-Piperidyl*)-1-*aminopropionyl*-DL-*leucine methyl ester* (XIV). The carbobenzyloxy groups were removed from (XIII) (0·15 g.) by catalytic hydrogenation by the same method as was used in (XI). The dihydrochloride was hygroscopic and could not be crystallized. As removal of carbobenzyloxy groups by this method is essentially quantitative, the product was deemed pure enough for test without crystallization.

Dicarbobenzyloxy - 2 - (*4′* - *piperidyl*) - 1 - *aminopropionyl* - *p-aminobenzoic acid methyl ester* (XV). An ethereal solution of the acid chloride of (IX) prepared as already described from (IX) (1·0 g.) was added dropwise, with shaking, to a solution of 2 equiv. (0·7 g.) of methyl-*p*-aminobenzoate in dry $CHCl_3$ (20 ml.). After leaving for 18 hr. at 37°, crystalline methyl-*p*-aminobenzoate hydrochloride (0·38 g.) was re-

moved and the $CHCl_3$-ether solution washed successively with dilute acid, bicarbonate and water. Removal of the solvent left a syrup (0·8 g.) which was redissolved in ethyl acetate and boiled with activated charcoal. The product, a colourless viscous oil (0·5 g.), could not be crystallized and was used for the next stage without further purification.

2-(4′-*Piperidyl*)-1-*aminopropionyl*-p-*aminobenzoic acid* (XVI). The acylated peptide ester (XV) (0·5 g.) was hydrolysed in a mixture of dioxane (5 ml.) and N-NaOH (5 ml.). After 30 min. at room temperature the mixture was acidified and diluted with water; the oil which separated was extracted into $CHCl_3$. The $CHCl_3$ was removed *in vacuo* and the residue hydrogenated in the presence of palladium black (100 mg.) and 2 equiv. of HCl. The dihydrochloride dihydrate of (XVI) crystallized as needles (0·1 g.), m.p. 190–192° (decomp.), when ether was added slowly to a methanolic solution of the product of hydrogenation. (Found: C, 45·0; H, 7·0; N, 10·7. $C_{15}H_{21}O_3N_3.2HCl.2H_2O$ requires C, 45·0; H, 6·8; N, 10·5%. Loss in weight at 105°, 8·4%. $2H_2O$ requires 9·0%.)

2-(3′:3′:3′-*Trichloro*-2′-*hydroxypropyl*)*pyridine hydrochloride* (XVII). The method used was based on earlier methods described by Einhorn & Liebrecht (1887), Löffler & Kaim (1909) and Tullock & McElvain (1939). Anhydrous chloral (110 ml.) was mixed with α-picoline (130 ml.) and dry amyl acetate (200 ml.). The mixture was heated for 14 hr. at 130° and then steam distilled to remove solvent and excess α-picoline. The non-volatile residue was decolorized with charcoal and concentrated *in vacuo*. After drying thoroughly over H_2SO_4, the product was crystallized from ethanol as needles, m.p. 200–201° (120 g.) (cf. Tullock & McElvain, 1939).

2-(2′-*Piperidyl*)*propionic acid hydrochloride* (XVIII). 2-(2′-Pyridyl)acrylic acid was prepared by the method of Löffler & Kaim (1909); the acrylic acid was reduced by the method used for the isomeric acid (III) and the product (XVIII) crystallized from aqueous acetone as the hydrochloride, m.p. 191–192°. The yield in the reduction was almost quantitative and gave none of the difficulties reported for other methods (Tullock & McElvain, 1939; Löffler & Kaim, 1909). (Found: C, 49·5; H, 8·3; N, 7·1. $C_8H_{15}O_2N.HCl$ requires C, 49·6; H, 8·3; N, 7·2%.)

Benzoyl-2-(2′-*piperidyl*)*propionic acid* (XIX). The method used for the isomeric compound (IV) was applied successfully to (XVIII) and the product crystallized from ethanol as needles, m.p. 158°. (Found: C, 69·0; H, 7·3; N, 5·7. $C_{15}H_{19}O_3N$ requires C, 69·0; H, 7·3; N, 5·4%.)

p-*Toluenesulphonyl*-2-(2′-*piperidyl*)*propionic acid* (XX). Tosylation was effected by the method of Thomas & Goerne (1919). A solution of (XVIII) (2 g.) in N-NaOH (25 ml.) was shaken vigorously with *p*-toluenesulphonyl chloride (2·5 g.) for 3 hr. Unchanged acid chloride was removed by filtration and the filtrate acidified. The precipitate was crystallized from 80% ethanol, prisms, m.p. 108–109° (1·0 g.) (Found: C, 57·6; H, 7·0; N, 4·7. $C_{15}H_{21}O_4NS$ requires C, 57·9; H, 6·8; N, 4·5%.)

MICROBIOLOGICAL RESULTS

When tested *in vitro* against *Streptococcus haemolyticus* (in Hartley's digest broth), *Staphylococcus aureus* (in medium of Strauss, Dingle & Finland, 1941) and *Escherichia coli* (in medium of MacLeod, 1940, supplemented with casein), analogues of lysine were found to be slightly antibacterial. Furthermore, the antibacterial activity of a simple analogue (β-4-piperidylalanine) was enhanced by its incorporation in peptide linkage with other amino-acids as indicated in Table 1.

Table 1. *Minimum inhibitory concentrations* (M) *of lysine analogues as inhibitors of bacterial growth*

Compound	Haemolytic streptococci		*Staph. aureus*		*Esch. coli*	
	Blood	Broth	Broth	Synthetic	Broth	Synthetic
1:6-Diaminohexane-1-carboxylic acid mono HCl	—	10^{-3}	$>10^{-2}$	—	$>10^{-2}$	—
2(4′-Piperidyl)-1-aminopropionic acid dihydrochloride	2×10^{-2}	8×10^{-3}	$>4\times10^{-2}$	2×10^{-2}	$>4\times10^{-2}$	$>4\times10^{-2}$
2(4′-Piperidyl)-1-aminopropionamide dihydrochloride	—	4×10^{-3}	$>4\times10^{-3}$	—	$>4\times10^{-3}$	—
2(4′-Piperidyl)-1-aminopropionyl-*p*-aminobenzoic acid dihydrochloride	—	$1{\cdot}5\times10^{-3}$	$>6\times10^{-3}$	6×10^{-3}	$>6\times10^{-3}$	$>6\times10^{-3}$
2(4′-Piperidyl)-1-aminopropionyl-DL-leucine methyl ester dihydrochloride	—	$1{\cdot}5\times10^{-3}$	$>1{\cdot}5\times10^{-3}$	—	$>1{\cdot}5\times10^{-3}$	—

DISCUSSION

At the time (1946) when the present investigation was planned some analogues of amino-acids were already known to exhibit limited antibacterial action: thus Fildes (1941) showed that indole-acrylic acid, an analogue of tryptophan, inhibited growth of *Esch. coli* at a concentration of 8×10^{-3}M; the effect was reversed by tryptophan. McIlwain (1941) had shown that α-aminosulphonic acids had some growth inhibitory action; Harris & Kohn (1941) had reported inhibition of growth of *Esch. coli* by ethionine; Roblin, Lampen, English, Cole & Vaughan (1945) had found that methoxinine, the oxygen analogue of methionine, inhibited *Esch. coli* and *Staph. aureus* in synthetic media; du Vigneaud, McKennis, Simmonds, Dittmer & Brown (1945) had shown that β-2-thienyl-alanine acted as a phenyl-

alanine antagonist, and Waelsh, Owades, Miller & Borek (1946) had studied methionine sulphoxide, methionine sulphone and benzylhomocysteine sulphoxide as antimetabolites of glutamic acid.

While the present investigation was under way the published knowledge of amino-acid analogues as growth inhibitors was augmented by a paper by Mitchell & Nieman (1947) on halogenated phenylalanines and tyrosines, of which 3-fluoro-DL-phenylalanine and 3-fluoro-L-tyrosine were the most active; by a communication from Elks, Hems & Ryman (1948) on α-amino acids with longer than normal aliphatic chains which were ineffective as growth inhibitors, and by a paper by Elliott, Fuller & Harington (1948) on alanines substituted in the β-position with pyridine, quinoline and basic derivatives of benzene. Significant inhibitions were observed only with β-ω-amino-*p*-tolylalanine, β-6-methoxyquinolyl-4-alanine and β-pyridyl-4-alanine; these compounds inhibited growth of *Strep. pyogenes* in broth at concentrations of 2×10^{-3}, $1{\cdot}5 \times 10^{-3}$ and 8×10^{-3}M respectively. Other substituted alanines showing significant action as inhibitors of bacterial growth were β-2-furylalanine and β-2-pyrrolealanine (Clark & Dittmer, 1948; Herz, Dittmer, and Cristol, 1948).

Without comparative tests, using the same strain of organism and the same batch of culture medium, it is impossible to make any comparison in absolute terms between the antibacterial efficacy of amino-acid analogues prepared in different laboratories. If any conclusion can be drawn from the experimental evidence available, it is that, as possible chemotherapeutic agents, essential amino-acid analogues do not offer great promise. The results obtained in the present investigation (Table 1) indicate, however, that in our series antibacterial activity was increased when an analogue of lysine (β-4-piperidyl alanine) was combined in peptide linkage with other amino-acids. This led to the abandonment of the present work, and to a study of synthetic peptides as possible antibacterial compounds; the results obtained will be reported in future papers.

SUMMARY

1. The reasons for synthesizing analogues of lysine as potential chemotherapeutic agents are discussed.

2. The following analogues have been synthesized and tested as inhibitors of bacterial growth against *Streptococcus haemolyticus*, *Staphylococcus aureus* and *Escherichia coli*: (1) 1:6-diaminohexane-1-carboxylic acid (homolysine); (2) 2-(4′-piperidyl)-1-aminopropionic acid (β-4-piperidyl alanine); (3) 2-(4′-piperidyl)-1-aminopropionyl-*p*-aminobenzoic acid; (4) 2-(4′-piperidyl)-1-aminopropionyl-DL-leucine methyl ester.

3. No striking growth inhibitory properties were encountered but increased inhibitory action was apparent when (2) was incorporated in peptide linkage with *p*-aminobenzoic acid and DL-leucine.

We are indebted to Dr A. T. Fuller for the biological results given in Table 1.

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The Synthesis of Peptides Related to Gramicidin S and the Significance of Optical Configuration in Antibiotic Peptides

1. TRIPEPTIDES

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In a previous communication (Harris & Work, 1950) we reported that an amino-acid analogue which was not itself antibacterial might acquire antibacterial properties when joined in peptide linkage with other amino-acids.

Repeated demonstration of the occurrence of amino-acids of 'unnatural' (D) configuration in antibiotic polypeptides such as gramicidin (Lipmann, Hotchkiss & Dubos, 1941), tyrocidine (Gordon, Martin & Synge, 1943), gramicidin S (Synge, 1945), aerosporin (Jones, 1948), and bacitracin (Barry, Gregory & Craig, 1948), suggested that antibiotic activity might be dependent upon the occurrence in such peptides of amino-acid analogues (viz. D-amino-acids) which, although harmless in themselves, became antibacterial when incorporated in a suitable peptide sequence. The presence of D-penicillamine among the hydrolysis products of penicillin, and the subsequent demonstration by du Vigneaud, Carpenter, Holley, Livermore & Rachele (1946) that antibiotic penicillin could be synthesized from D-penicillamine, whereas the isomeric compound from L-penicillamine was inactive, seemed at first sight to lend support to this view.

To test such a theory it did not seem sufficient to link any one of the D-amino-acids occurring in antibiotic peptides with a random selection of L-amino-acids, and the choice of a suitable series of synthetic peptides was governed by the knowledge that in only one antibiotic peptide, namely, gramicidin S, was the probable sequence of amino-acids known.

On the basis of degradative studies, Consden, Gordon, Martin & Synge (1947) have suggested that gramicidin S is a cyclic pentapeptide or a cyclic decapeptide with the following sequence of amino-acid residues: L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl-L-valyl- (Fig. 1). As the only amino-acid of 'unnatural' configuration, phenylalanine is indicated in Fig. 1 by the projection of the benzyl side chain below the line of the peptide 'backbone'. We sought to examine the significance of optical configuration by synthesis, in the first instance, of the tripeptide fragment enclosed by the dotted lines in Fig. 1. The corresponding tripeptide with all three amino-acids in the L configuration was also synthesized as a control.

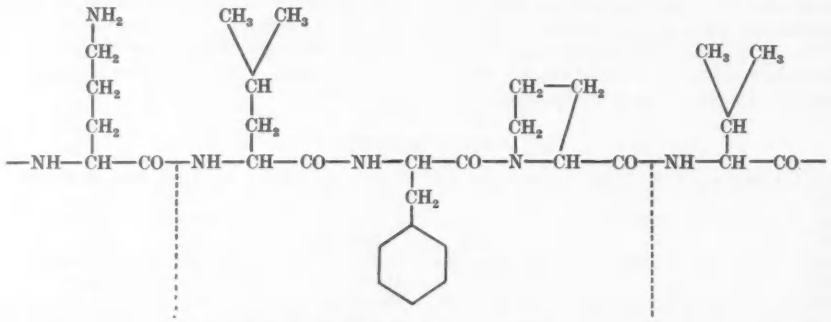

Fig. 1. Amino-acid sequence in gramicidin S.

Synthetic methods

For the syntheses of the optically isomeric tripeptides, carbobenzyloxy derivatives of appropriate amino-acids were used according to the general pro-

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cedure of Bergmann & Zervas (1932). Difficulties were encountered in the syntheses of dipeptide esters containing proline, and although *carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester* and *carbobenzyloxy-D-phenylalanyl-L-proline methyl ester* were obtained by the azide method, yields were low and the products were not crystallized. It is relevant to note that Synge (1948) also reported difficulty in the isolation of pure dipeptides containing proline; his products were amorphous and analytical figures departed appreciably from the expected values.

In view of the tendency of proline dipeptides to form diketopiperazines (Abderhalden & Nienburg, 1933; Smith & Bergmann, 1944), and as satisfactory characterization of intermediates was considered desirable, it was decided to modify the order of coupling. In the preparation of carbobenzyloxy-L-leucyl-D-phenylalanine ester the acid chloride and azide methods of coupling were compared; a higher yield and a purer product was obtained by the azide method and this was accordingly the method of choice whenever applicable. The azide from carbobenzyloxy-L-leucyl-D-phenylalanyl hydrazide coupled smoothly with L-proline methyl ester to give *carbobenzyloxy - L - leucyl - D - phenylalanyl - L - proline methyl ester* in 85% yield; the corresponding carbobenzyloxy-L-L-L-tripeptide ester, prepared by coupling carbobenzyloxy-L-leucyl azide with L-phenylalanyl-L-proline ethyl ester, was obtained in inferior yield as an amorphous solid. The identity and purity of the tripeptide esters was checked by elementary analyses, by paper chromatography of the tripeptide esters themselves, and by comparison of their acid hydrolysates with an artificial mixture of the component amino-acids.

EXPERIMENTAL

Amino-acids. Leucine and phenylalanine were synthesized by the acetamidomalonic ester synthesis of Albertson & Archer (1945), and resolved through the brucine salts of their formyl derivatives by the classical procedures of Fischer (1906). L-Proline was isolated from a protein hydrolysate by the rhodanilate method of Bergmann (1935).

Carbobenzyloxy-L-phenylalanyl hydrazide. L-Phenylalanine methyl ester was prepared according to the general method of Fischer (1901). The methyl ester (13·0 g.) in dry ether (100 ml.) was cooled to $-10°$ and an ethereal solution of carbobenzyloxy chloride (6·2 g., 0·5 equiv.) was added dropwise, with stirring, during 45 min. The reaction mixture was left overnight at room temperature and the precipitated L-phenylalanine methyl ester hydrochloride recovered by filtration. Carbobenzyloxy-L-phenylalanine methyl ester was obtained as an oil (10·0 g.) after removal of solvent from the filtrate. This oil was mixed in methanol with an excess of 50% hydrazine hydrate and left for 24 hr. at room temperature. *Carbobenzyloxy-L-phenylalanine hydrazide* was precipitated on addition of water and collected by filtration. The pure hydrazide (7·9 g.) was obtained as needles, m.p. 168°, from methanol. (Found: C, 65·4; H, 6·2; N, 13·6. $C_{17}H_{19}O_3N_3$ requires C, 65·2; H, 6·1; N, 13·4%.)

Carbobenzyloxy-D-phenylalanine hydrazide. D-Phenylalanine hydrochloride (25 g.) yielded carbobenzyloxy-D-phenylalanine methyl ester (20 g.). The ester was converted to *carbobenzyloxy-D-phenylalanine hydrazide* by the method previously used for the L-isomer. The hydrazide crystallized from ethanol as needles, m.p. 169–170° (16·5 g.). (Found: C, 65·6; H, 6·4; N, 13·5. $C_{17}H_{19}O_3N_3$ requires C, 65·2; H, 6·1; N, 13·4%.)

Carbobenzyloxy - L - phenylalanyl - L - proline ethyl ester. L-Phenylalanine hydrazide (6·7 g.) in a mixture of glacial acetic acid (50 ml.) and 2N-HCl (100 ml.) was cooled to 0° and a solution of $NaNO_2$ (1·1 g.) in water (10 ml.) added dropwise to the stirred mixture during 10 min. The precipitated azide was extracted into cold ether (100 ml.) and washed with ice-cold water, with cold saturated $NaHCO_3$ solution and again with water. The azide solution was dried quickly over Na_2SO_4 and added during 20 min. to a cooled solution of L-proline ethyl ester (4·0 g.) in dry ether (100 ml.). After 24 hr. at 0° the solution was washed with dilute acid to remove proline ester, then with $NaHCO_3$ solution, and finally with water. Removal of the solvent *in vacuo* left a pale yellow solid (8·0 g.) which was not obtained in a crystalline form. (Found: C, 67·5; H, 6·3; N, 6·8. $C_{24}H_{28}O_5N_2$ requires C, 67·9; H, 6·6; N, 6·6%.)

L-Phenylalanyl-L-proline ethyl ester. Removal of the carbobenzyloxy group from carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester was readily accomplished by application of the catalytic hydrogenation procedure of Bergmann & Zervas (1932). *L-Phenylalanyl-L-proline ethyl ester hydrochloride* crystallized from ethanol as extremely hygroscopic needles, m.p. (sealed tube) 174° (decomp.). Owing to rapid uptake of water, no satisfactory analytical results could be obtained for this hydrochloride.

Carbobenzyloxy - L - leucyl - L - phenylalanyl - L - proline ethyl ester. Carbobenzyloxy-L-leucyl hydrazide was prepared from L-leucine by the method of Bergmann, Zervas, Fruton, Schneider & Schleich (1935). The hydrazide (2·0 g.) was converted to the azide and allowed to react with L-phenylalanyl-L-proline ethyl ester (2·7 g.). The method of handling was similar to that already employed in the synthesis of carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester. The product, a yellow gum (1·9 g.), was dissolved in ethyl acetate and precipitated with ligroin. Repetition of this operation four times yielded a product (1·5 g.) which was not obviously crystalline but which nevertheless melted rather sharply (134–136°). Analysis and paper chromatography of a hydrolysate left no doubt that it was *carbobenzyloxy-L-leucyl-L-phenylalanyl-L-proline ethyl ester.* (Found: C, 67·2; H, 7·3; N, 8·0. $C_{30}H_{39}O_6N_3$ requires C, 67·1; H, 7·3; N, 7·8%.)

L-Leucyl-L-phenylalanyl-L-proline ethyl ester. The carbobenzyloxy derivative (1·2 g.) from the previous experiment was successfully hydrogenated in ethanol in the presence of 1 equiv. of H_2SO_4. Removal of acid and solvent left an oil (0·95 g.) which could not be crystallized. Provisional identification of the product as *L-leucyl-L-phenylalanyl-L-proline ethyl ester* was achieved by paper chromatography.

Carbobenzyloxy-L-leucyl-D-phenylalanine ethyl ester. (*Acid chloride method.*) Carbobenzyloxy-L-leucine (6·2 g.) in dry ether (30 ml.) was allowed to react at 0° with PCl_5 (4·9 g.). When the reaction was complete, ether was removed and the residual oil washed with three 50 ml. portions of dry light petroleum. The petroleum-insoluble acid chloride was dissolved in dry $CHCl_3$ (25 ml.) and half of this solution was added, with stirring, to a cooled solution ($-10°$) of D-phenylalanine ethyl ester (4·5 g.) in dry $CHCl_3$ (25 ml.); after 5 min.

$KHCO_3$ (2·32 g.) was added in the minimum volume of water and followed by the remainder of the acid chloride. The mixture was stirred and the temperature held below 0° for 30 min. and at 15° for another hour. The $CHCl_3$ solution was washed successively with dilute acid and dilute bicarbonate and dried. Removal of solvent left carbobenzyloxy-L-leucyl-D-phenylalanine ethyl ester which crystallized from a mixture of ethyl acetate and light petroleum as needles (5·0 g.), m.p. 105°. (cf. Synge, 1948). (Found: C, 67·8; H, 7·2; N, 6·2. $C_{25}N_{32}O_5N_2$ requires C, 68·1; H, 7·3; N, 6·4%.)

L-Leucyl-D-phenylalanine ethyl ester. The carbobenzyloxy peptide as prepared above (1·0 g.) was hydrogenated in methanol containing 1 equiv. HCl. The carbobenzyloxy group was readily removed and *L-leucyl-D-phenylalanine ethyl ester hydrochloride* crystallized from ethyl acetate-ethanol as plates (0·8 g.), m.p. 216°. (Found: C, 59·6; H, 7·7; N, 8·0. $C_{17}H_{26}O_3N_2$.HCl requires C, 59·6; H, 7·9; N, 8·2%.)

Carbobenzyloxy - L - leucyl - D - phenylalanine methyl ester. (Azide method.) Carbobenzyloxy-L-leucyl hydrazide (18 g.) was converted to the azide and allowed to react with phenylalanine methyl ester (16·8 g.). The method was essentially that employed already in the synthesis of carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester. The product, *carbobenzyloxy-L-leucyl-D-phenylalanine methyl ester* (20·8 g.) crystallized as needles, m.p. 110–112°, from ether. (Found: C, 67·4; H, 7·1; N, 6·7. $C_{24}H_{30}O_5N_2$ requires C, 67·6; H, 7·1; N, 6·6%.) Comparison of this yield (77%) with that obtained by the acid chloride method (48%) indicated that the azide method was to be preferred.

Carbobenzyloxy-L-leucyl-D-phenylalanyl hydrazide. The methyl ester from the above preparation (19·0 g.) was converted to the hydrazide by treatment in methanol, with an excess of 90% hydrazine hydrate. *Carbobenzyloxy-L-leucyl-D-phenylalanyl hydrazide* (16·6 g.) crystallized from ethyl acetate as needles, m.p. 169. (Found: C, 64·5; H, 7·2; N, 13·2. $C_{23}H_{30}O_4N_4$ requires C, 64·8; H, 7·1; N, 13·2%.)

Carbobenzyloxy-L-leucyl-D-phenylalanyl-L-proline methyl ester. Carbobenzyloxy-L-leucyl-D-phenylalanyl hydrazide (16·0 g.) was converted to azide and reacted with proline methyl ester (12 g.) by the usual azide method, the only modifications being substitution of ethyl acetate as solvent in place of ether and allowance of 48 hr. at 0° for completion of the reaction. Excess proline ester was recovered as the hydrochloride (6·6 g.). *Carbobenzyloxy-L-leucyl-D-phenylalanyl-L-proline methyl ester* (19·0 g.) was a solid of doubtful crystallinity melting over the range 40–42° ($[\alpha]_D^{22°}$, −42·2° in methanol, *c*, 2·7). Comparison of an acid hydrolysate of this peptide with an artificial mixture of the constituent amino-acids on a paper chromatogram, coupled with almost quantitative reduction of a sample of carbobenzyloxy tripeptide ester to a crystalline tripeptide ester hydrochloride, left no doubt that the desired product had been obtained in an almost pure state in a yield of 85% of theory; analysis of the amorphous solid confirmed this view. (Found: C, 65·9; H, 7·1; N, 8·5. $C_{32}H_{37}O_6N_3$ requires C, 66·5; H, 7·1; N, 8·1%.)

L-Leucyl-D-phenylalanyl-L-proline methyl ester. The carbobenzyloxy tripeptide ester (1·6 g.) from the previous experiment was hydrogenated with palladium black in methanol in the presence of 1 equiv. of HCl. Evolution of CO_2 was complete in 2 hr. *L-Leucyl-D-phenylalanyl-L-proline methyl ester hydrochloride*, white hygroscopic plates, m.p. 240° (decomp.), crystallized from a mixture of ethanol and ether; $[\alpha]_D^{20°}$ − 38·9° in methanol (*c*, 2·0). (Found: C, 58·9; H, 7·5; N, 10·0. $C_{21}H_{31}O_4N_3$.HCl requires C, 59·2; H, 7·5; N, 9·9%.)

MICROBIOLOGICAL RESULTS

The optically isomeric tripeptide ester hydrochlorides, together with the intermediate dipeptide esters, were tested *in vitro* against *Staphylococcus aureus*, *Streptococcus haemolyticus* and *Escherichia coli* (media: see Harris & Work, 1950), and the results obtained are given in Table 1. It was not considered necessary to remove the ester group before testing since esters are readily hydrolysed by living cells.

Table 1. *Minimum inhibitory concentrations* (M) *of synthetic peptide esters as inhibitors of bacterial growth*

Compound	*Esch. coli* (synthetic medium)	*Staph. aureus* (synthetic medium)	*Strep. haemolyticus* (broth)
D-Phenylalanyl-L-proline methyl ester	3×10^{-2}	2×10^{-2}	3×10^{-2}
L-Phenylalanyl-L-proline ethyl ester	2×10^{-2}	4×10^{-2}	$1{\cdot}5 \times 10^{-2}$
L-Leucyl-D-phenylalanine methyl ester	5×10^{-3}	2×10^{-3}	1×10^{-3}
L-Leucyl-D-phenylalanyl-L-proline methyl ester	7×10^{-3}	5×10^{-3}	$1{\cdot}5 \times 10^{-3}$
L-Leucyl-L-phenylalanyl-L-proline ethyl ester	$>7 \times 10^{-3}$	6×10^{-3}	1×10^{-3}

DISCUSSION

It is apparent from Table 1 that synthetic L-leucyl-D-phenyl-alanyl-L-proline ester is slightly antibacterial, but this activity is not peculiarly associated with the occurrence of D configuration, since the optically isomeric L-leucyl-L-phenylalanyl-L-proline ester is equally active.

This result is in general agreement with the findings of other investigators which have been published while the present work was in progress. In view of the isolation of D-leucine and D-valine from gramicidin (Dubos) hydrolysates, and since the intact gramicidin molecule contains no free primary amino groups, Fling, Minard & Fox (1947) synthesized prolyl derivatives of leucine and valine. No antipodal specificity was encountered despite the fact that the free D-amino-acids had shown some inhibition of bacterial growth under similar conditions (Fox, Fling & Bollenback, 1944; Fling & Fox, 1945). All four diastereoisomeric leucylleucines have also been synthesized and tested, but not one had antibacterial activity equal to that of D-leucine itself (Fox, Kobayashi, Melvin & Minard, 1948). The high content of D-leucine and L-tryptophan in gramicidin

led Fruton (1948) to synthesize the diketopiperazine from D-leucine and L-tryptophan, but no antibacterial activity was encountered.

These findings indicate that growth inhibitory properties cannot be correlated with the mere presence of D-amino-acids in peptide linkage. At the outset of the present investigation it was considered likely that, as an additional requirement, the D-amino-acid should be flanked by a suitable pair of amino-acids; it was supposed that when this requirement was met optically selective antibiotic action might appear. A tripeptide possessing the D-amino-acid present in gramicidin S, flanked by the same two amino-acids as in the natural antibiotic peptide has, however, failed to show selective toxicity.

All the available evidence now supports the view expressed by Work (1948) that peptide antibiotics containing D-amino-acids are active, not because they have this character in common, but rather by virtue of the individual structure of the peptide.

In order to eliminate as far as possible the complicating factors of molecular size, and number and type of polar groups per molecule, it seemed desirable to extend the present investigation by the syntheses of appropriate pentapeptides containing the complete amino-acid sequence of gramicidin S; the results of this investigation will be reported in a future communication.

SUMMARY

1. Dipeptides and tripeptides containing D-phenylalanine have been synthesized and compared as growth inhibitors with optically isomeric peptides containing L-phenylalanine.

2. The amino-acids in these peptides were arranged in the same sequence as in gramicidin S.

3. Limited antibiotic activity was observed with synthetic peptides, but no difference in activity was found between a tripeptide containing D-phenylalanine and one containing L-phenylalanine.

4. The significance of these results in relation to the origin of antibiotic activity in peptides is discussed.

We are indebted to Dr A. T. Fuller for the biological results summarized in Table 1.

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The Steady State, a Test for Electrophoretic Homogeneity

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The distribution of the ion concentrations across the boundaries formed by a solution of a homogeneous protein changes during electrophoresis. The tendency is for the boundary in one limb of the U-tube to become narrower and for that in the other to become broader, mainly on account of the differences in conductivity on both sides of each boundary (Longsworth & MacInnes, 1940), but also because the mobility changes with the ionic strength. These effects may be enhanced or counteracted by changes in pH (Longsworth, 1947). If the ascending boundary of a leading ion has the tendency to become narrower, and this is generally the case with proteins at a pH removed from the isoelectric point, its behaviour during migration may be followed for an unlimited period of time, provided a countercurrent of buffer

solution of constant composition can be kept up through the U-tube. The factors responsible for the spreading of initially sharp boundaries have been discussed by Ogston (1946). Spreading can be due to thermal diffusion or to electrical heterogeneity. Considering a complex electrolyte, such as a protein, which is present in a number of ionic forms of different mobility, Ogston (1946) could show that the spreading of the boundary is formally equivalent to diffusion, except that it increases as the square of the potential gradient. Since, for a given potential gradient, the rate at which the boundary becomes broader decreases with increasing time while the tendency to sharpen remains constant being dependent only on the difference between the migration velocities of the protein ions at either side of the boundary, a steady state at which spreading and sharpening balance should be approached. The mathematical solution for the steady state occurring in the differential moving-boundary method for transport numbers of strong electrolytes has been given by Longsworth (1943). In the following the theoretical shape of the boundary and the possibility of experimentally reaching and maintaining such a steady state with a protein will be examined.

THEORETICAL

Notation. V_0 = migration velocity of the protein ions at infinitely low concentration in the buffer solution; V = actual migration velocity of the protein ions; V_s = migration velocity of the boundary in the steady state; C = concentration of protein in g./100 ml.; C_0 = concentration of protein in the adjusted solution below the boundary; K, K_1, K_2, etc. = coefficients; x = vertical position in the U-tube; t = time; D' = apparent diffusion contsant.

Derivation. Equation (1), which had been proposed as a first approximation valid for low colloid concentrations (Hoch, 1948), can be extended as in (2):

$$V = V_0(1 + KC), \tag{1}$$

$$V = V_0(1 + KC + KK_1C^2 + KK_1K_2C^3 + \ldots). \tag{2}$$

This series converges if KC, K_1C, K_2C, etc. <1. For the particular case of serum albumin in phosphate buffer of pH 8, $I = 0{\cdot}1$, it has been shown experimentally that K is about 0·06 at a protein concentration of 1 g./100 ml. and theoretically, on the basis of Svensson's (1946) boundary equation, that the variation in K from $C = 0$ to $C = 1$ g./100 ml. is 11% or less (Hoch, 1948; Hoch-Ligeti & Hoch, 1948). This is equivalent to $K_1 = 0{\cdot}11$ (or less) if the members of the series in (2) containing K_2, K_3, etc., are neglected. Equation (2) then becomes

$$V = V_0(1 + KC + KK_1C^2) = V_0(1 + KC(1 + K_1C)). \tag{3}$$

The following derivation of the shape of the curve of dc/dx against x will be based on equation (3) or on equation (1). The results should therefore be considered as approximations valid for K_2C, K_3C, etc. $\ll 1$.

If a boundary of a homogeneous protein moves in the steady state its velocity of migration is $V_s = V_0(1 + KC_0 + KK_1C_0^2)$. The amount of protein, per unit area of cross-section transported with the velocity $V = V_0(1 + KC + KK_1C^2)$ across a plane in the boundary in excess of that amount transported across a neighbouring plane with the velocity

$$V = V_0(1 + K(C + dC) + KK_1(C + dC)^2)$$

is

$$V_0(KdC + 2KK_1CdC)\,Cdt.$$

The change in concentration therefore in the lamina bounded by the planes, with reference to a co-ordinate system moving with the velocity

$$V = V_0(1 + KC + KK_1C^2)$$

is

$$(dC)_1 = V_0(K + 2KK_1C)\,C\frac{dC}{dx}\,dt.$$

In the co-ordinate system moving with the velocity V_s, any point on the curve of C against x, which moves with a velocity $C < C_0$, lags behind by the distance

$$[V_0K(C_0 - C) + V_0KK_1(C_0^2 - C^2)]\,dt$$

corresponding to a change in concentration

$$(dC)_2 = [V_0K(C_0 - C) + V_0KK_1(C_0^2 - C^2)]\,dt\frac{dC}{dx}.$$

$(dC)_1 - (dC)_2$ is the change in concentration at a point in the system moving with the velocity V_s. Hence the change in concentration per unit time, due to the sharpening effect

$$\frac{dC}{dt} = V_0(K + 2KK_1C)\,C\frac{dC}{dx} - [V_0K(C_0 - C) + V_0KK_1(C_0^2 - C^2)]\frac{dC}{dx}$$

$$= -V_0K(C_0 + K_1C_0^2 - 2C - 3K_1C^2)\frac{dC}{dx}.$$

At any point the change in concentration per unit time, due to spreading, is given by

$$\frac{dC}{dt} = -D'\frac{d^2C}{dx^2},$$

where D' covers the effects of thermal diffusion, the spreading through the presence of different interconvertible ionic forms, mechanical disturbance and the effects brought about by the process of compensation. It is assumed to be constant.

In the steady state, the spreading and sharpening effects balance each other

$$-D'\frac{d^2C}{dx^2} = -V_0K(C_0 + K_1C_0^2 - 2C - 3K_1C^2)\frac{dC}{dx}. \tag{4}$$

Integrating once

$$\frac{dC}{dx} = \frac{V_0K}{D'}[(C_0 + K_1C_0^2)\,C - C^2 - K_1C^3] + m. \tag{5}$$

Since $dC/dx = 0$ for $C = 0$ and $C = C_0$ it follows that $m = 0$. Rearranging,

$$\frac{dC}{C\left(C^2 + \frac{1}{K_1}C - \frac{1}{K_1}C_0 - C_0^2\right)} = -\frac{V_0KK_1}{D'}dx,$$

$$\frac{dC}{C_1C_2C} + \frac{dC}{C_1(C_1 - C_2)(C - C_1)} - \frac{dC}{C_2(C_1 - C_2)(C - C_2)} = -\frac{V_0KK_1}{D'}dx,$$

where C_1 and C_2 are the roots of

$$C^2 + \frac{1}{K_1}C - \frac{1}{K_1}C_0 - C_0^2 = 0; \quad C_1 = 0, \quad C_2 = -\left(C_0 + \frac{1}{K_1}\right).$$

Integration gives

$$-\frac{1}{C_0\left(C_0+\frac{1}{K_1}\right)}\log C+\frac{1}{C_0\left(2C_0+\frac{1}{K_1}\right)}\log(C_0-C)$$
$$-\frac{1}{\left(C_0+\frac{1}{K_1}\right)\left(2C_0+\frac{1}{K_1}\right)}\log\left(C+C_0+\frac{K_1}{1}\right)$$
$$=-\frac{V_0KK_1}{D'}(x+\text{const.}) \quad (6)$$

If K_1C_0 can be neglected relative to unity, equation (5) becomes

$$\frac{d^2C}{dx^2}=\frac{V_0K}{D'}(C_0-2C)\frac{dC}{dx}. \quad (7)$$

Integrated once

$$\frac{dC}{dx}=\frac{V_0K}{D'}(C_0C-C^2)+m, \quad (8)$$

This equation is formally equivalent to Longsworth's (1943) equation $\frac{dc}{dh}=-\frac{a_2i(\Delta c)^2}{4FD}\Big/\cosh^2\frac{a_2i\Delta c}{2FD}h$.

With the protein boundaries considered here the buffer salt concentration is greater above the boundary than it is below. If such a boundary, formed between the adjusted solution and the buffer, is left to diffuse without migration, the diffusion of the buffer salt, being faster than that of the protein, will cause the formation of two gravitationally unstable regions; the salt concentration in the region above the protein boundary will be smaller than that of the bulk of the buffer solution above it, and the salt concentration in the region below the protein boundary will be greater than that of the adjusted solution underneath. One must assume that convection will result, and that the composition of the solutions on either side of the protein boundary will remain unchanged in the regions where the density gradient due to the change in the protein concentration is sufficiently small. Consequently, the variation in salt concentration across the boundary will tend towards becoming linear. On migration the distributions of the gradients of the buffer ion and the protein concentrations will approach each other in shape. The protein concentration curve, which has been derived without considering the effects just described, will be distorted from a sigmoid towards a linear form. This should result in a decrease of the protein concentration gradients in the centre of the boundary and an increase of the gradients at the tails. It is clear, therefore, that the spreading of the boundary arising from the diffusion of the buffer ions cannot be expressed by an additive term in the apparent diffusion constant D'. The calculation of the gradient curve with the help of a fictitious diffusion constant, together with the qualitative consideration of the effects of the diffusion of the buffer salt ions, serves to indicate the direction in

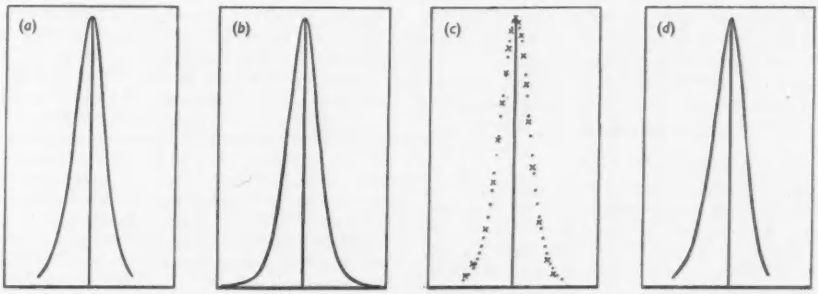

Fig. 1. Theoretical shapes of boundary patterns. *a*, calculated from equations (5) and (6) with $C_0=0{\cdot}85$ and $K_1=0{\cdot}3$; the value for K_1 was arbitrarily chosen to represent an experimental possibility. *b*, calculated from equation (10). *c*, curves of *a* and *b* superimposed. *d*, calculated from equations (5) and (6) with $C_0=0{\cdot}85$ and $K_1=1{\cdot}0$. The units for both the abscissae and ordinates are arbitrary.

where $m=0$ as in equation (5); rearranging equation (8) and integrating

$$\left.\begin{aligned}\frac{dC}{C_0C}+\frac{dC}{C_0(C_0-C)}&=\frac{V_0K}{D'}dx,\\ \log\frac{C}{C_0-C}&=\frac{V_0KC_0}{D'}(x+n).\end{aligned}\right\} \quad (9)$$

If $x=0$ is chosen for $d^2C/dx^2=0$ and $dC/dx\neq0$, by inserting equation (8) in equation (7),

$$\frac{d^2C}{dx^2}=\left(\frac{V_0K}{D'}\right)^2(C_0-2C)(C_0-C)C=0;$$

this gives the solutions $C_1=0$, $C_2=C_0$, $C_3=C_0/2$. The maximum of the curve of dC/dx is at $C_3=C_0/2$. Inserting this value in (9) n becomes zero and

$$\frac{C}{C_0-C}=\exp\left(\frac{V_0KC_0}{D'}x\right) \quad \text{and} \quad C=C_0\frac{\exp\left(\frac{V_0KC_0}{D'}x\right)}{1+\exp\left(\frac{V_0KC_0}{D'}x\right)}.$$

Inserting this value for C in (8) we obtain

$$\frac{dC}{dx}=\frac{V_0KC_0^2}{D'}\frac{\exp\left(\frac{V_0KC_0}{D'}x\right)}{\left[1+\exp\left(\frac{V_0KC_0}{D'}x\right)\right]^2}. \quad (10)$$

which the experimental curves would be expected to deviate from the error curve.

Fig. 1 *a*, *b* shows the plots of equations (5) and (10). In order to compare the curves with respect to the variation in the width with the relative height, all curves were plotted on such scales as to make the heights and the areas under the peaks equal. As can be seen from equation (10) changes in C_0, V_0, D' and K affect only the width and the height but not the characteristic shape of the curve. Equation (5) gives a slightly asymmetrical curve which is less steep on the side towards the low protein concentration than on the other side. Both curves differ little from each other as seen in Fig. 1 *c*, where they are superimposed. Compared with a Gaussian error curve these curves are narrower at the top and wider at the bottom. Increase of K_1 leads to a greater asymmetry and a greater deviation from the error curve, but the effect is slight, as can be seen in Fig. 1 *d*, where $K_1 = 1{\cdot}0$ has been used. From equation (10) it follows that the width of the peak is inversely proportional to the migration velocity, i.e. the potential gradient, if all other conditions remain the same.

EXPERIMENTAL

Materials. (*a*) Oxyhaemoglobin (HbO_2). Normal human red cells were washed 4 times with 0·85% (w/v) NaCl, and lysed with 2–3 times their volume of water. To the clear supernatant was added 0·1 vol. of 0·33 M-Na_2HPO_4, the precipitate which formed was centrifuged (by centrifuging (Hoch, 1949) the turbid fast component described by Stern, Reiner & Silber (1945) was removed) and the supernatant diluted with buffer solution to the required concentration of HbO_2. (*b*) Crystallized bovine serum albumin prepared by Armour Laboratories, Chicago, Illinois. The author is indebted to Dr H. Gutfreund for a sample of this material. (*c*) Serum from a blood donor who had hypertension. The protein pattern was normal but for the albumin peak which remained single on prolonged electrophoresis.

Electrophoresis and diffusion measurements. The experiments were made in a Tiselius apparatus equipped with a cylindrical lens schlieren optical system (Thovert, 1914; Philpot, 1938; Svensson, 1939). A diagonal wire was used to produce the patterns for subsequent tracing. Some of the patterns were also recorded with a diagonal edge because of their superior appearance in print. The light passing through the U-tube was horizontal and parallel. The focusing, particularly that of the U-tube on the plate, was checked by observing the patterns of the sharp initial boundaries. They were symmetrical and their axes were vertical to the base line. An error curve fitted to a diffusion boundary pattern obtained under the same conditions also showed that the optical set up was satisfactory.

The experiments with HbO_2 were made in solutions of 0·01 M-Na_2HPO_4. The fact that the phosphate solution has a negligible buffering capacity has no bearing on the present experiments since any pH effects are taken care of in the determination of K made under the same conditions. The experiments with albumin were made in phosphate of pH 8, $I = 0{\cdot}1$. The conditions for the experiments with HbO_2 were chosen so as to give pronounced boundary anomalies and a relatively high migration velocity of the HbO_2.

All runs were carried on beyond the time during which the ascending boundary migrated the distance of one limb of the U-tube and a constant current of buffer solution was kept up in the direction opposite to that of the migration of the boundary. In order to prevent the Cl^- in the bottom of the electrode vessels from reaching the U-tube a layer of 5–10 times concentrated phosphate solution of the same pH was put on top of the solution of NaCl (Tiselius, 1938), and on this a layer of cotton wool (Svensson, 1947). In Exp. 1 a continuous current of buffer was maintained within the cathode vessel from the top towards the cotton-wool pad, in addition to the compensation current through the U-tube. At the end of this run which lasted 22 hr. (with an interruption of 11 hr.), the concentration of Cl^- in the phosphate solution taken from the U-tube was less than 0·0002 N, or 2% of the anion concentration of the phosphate solution used.

The relative change in migration velocity per unit concentration, K, was determined for HbO_2, as previously described for serum albumin (Hoch, 1948), by observing the migration velocity of a boundary of a dilute solution of HbO_2 of 0·1 or 0·05 g./100 ml. simultaneously with that of a boundary of a solution of 1·15 g./100 ml. The migration velocities at given currents were determined in the usual manner at the beginning and at the end of an experiment.

The concentrations of HbO_2 were determined by photoelectric measurements of light absorption in the visible region, and those of albumin by micro-Kjeldahl.

The diffusion constant (D) was determined under the conditions of the distribution of the buffer ions as obtaining at the ascending boundary. For example, in the case of HbO_2, a solution of 1 g./100 ml. was electrolysed till the greater part of the ascending limb was taken up by the solution forming between the HbO_2 boundary and the δ boundary. A fresh boundary was then formed by the sharpening technique of Kahn & Polson (1947), as modified by Alberty (1948), immediately below the original boundary and left to diffuse at 0°. D was determined by the area maximum height method (A, $h_{max.}$) employing from five to seven readings distributed over a period of from 1 to 20 hr. The coefficient of variation of the individual readings from a straight line plot of $(A/h_{max.})^2$ against time was 1·4%.

Observation of boundaries. The changes in the form of a boundary are most easily detected by measuring the changes in the height of the peak and this method was used throughout. The tilt of the diagonal wire was kept constant except where the change in the height was greater than could be recorded with one tilt angle. The values were then calculated to refer to a constant tilt angle.

It is important to keep the boundary in the same place if small changes in the shape of the pattern are to be observed, otherwise the defects of the optical correction of the system may interfere. While in Exp. 1, the boundary was always kept near and photographed at a chosen position in the U-tube, in Exp. 2 the readings of the heights of the peaks were corrected by linear interpolation between the values obtained at different distances from the chosen position.

RESULTS

Heterogeneity of oxyhaemoglobin preparations from red cells

In addition to the components of the normal human erythrocyte described by Stern *et al.* (1945), two components faster than the oxyhaemoglobin were

found in fresh red-cell extracts. Fig. 2*a*, *b* shows the ascending patterns recorded in a run with an oxyhaemoglobin solution of 1·34 g./100 ml. The two faster components are present in quantities much smaller than appeared from the relative proportions of the areas underneath the peaks, because the similarity of their mobilities with that of the main component causes an increase in their concentration ahead of the main boundary to a multiple of their original concentration. With simplifying assumptions (Hoch-Ligeti & Hoch, 1948), including the absence of specific interaction between the components, a concentration of 2·5 % of the total was derived. These faster peaks could also be seen in patterns from a run with a solution of 1 g./100 ml. (from a different subject), but here their size was smaller (Fig. 2*d*). A calculation similar to that above gave a value of 1·5 % of the total. It is necessary to separate these peaks sufficiently before the oxyhaemoglobin boundary can be studied.

With solutions that have been kept in the cold for 2 weeks or longer, a small peak occurs between the main peak and the slower peak (Fig. 2*c*), but in fresh preparations this is barely detectable.

The steady state

Haemoglobin. Fig. 3*a* shows a plot of the height of an oxyhaemoglobin boundary peak against the time and distance of migration. A stationary state was reached after 430 min. and it was maintained for a further 230 min. After the boundary had been left to diffuse overnight, the stationary state was reached again after migrating for about 250 min. and was then maintained for 350 min. Fig. 2*e*, *f* shows the boundary after it had migrated 17 and 56 cm. respectively. The shape has hardly changed. The boundary was slightly more asymmetrical at the later stage.

In a second experiment similar results were obtained as seen in Fig. 3*b*. In this experiment no precautions had been taken to prevent the NaCl from reaching the U-tube except for the insertion of the cotton-wool pad. The steady state reached, after diffusion of the boundary had taken place overnight, gave a 17 % smaller height of the peak than was observed before the boundary was left to diffuse. The migration velocity (at 12·0 ma.) was found to be 12·5 % less at the end of the experiment than at the beginning, an indication of an increase in conductivity. At the end of the run the concentration of Cl^- in the supernatant phosphate solution from the ascending limb was about 0·002 N; this is 20 % of the anion concentration of the phosphate solution. The observed broadening of the boundary could thus be accounted for.

Serum albumins. Fig. 4*a* shows the changes in the height of the peak during 21 hr. of electrophoresis of a solution of crystallized bovine albumin. Some of the patterns are reproduced in Fig. 2*g–j*. The height of the peak diminished progressively except for a slight rise after the first period of diffusion (not recorded in the figure) and no steady state was reached. That the progressive spreading of the boundary could not have been due to a change in conductivity was ascertained by measuring the migration velocities at the beginning and at the end of the experiment. They were found to differ by less than 1 %. Therefore, although the boundary peak was symmetrical and

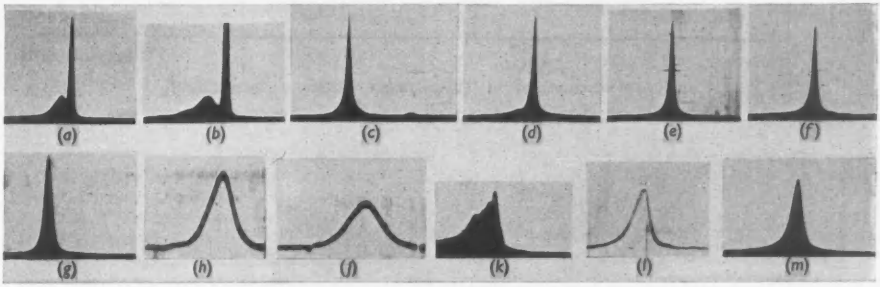

Fig. 2. Electrophoretic patterns of HbO_2 and of albumins. *a*, *b*, HbO_2, 1·34 g./100 ml.; *c–f*, 1·0 g./100 ml.; 0·01 M-Na_2HPO_4, 16 V./cm.; times of migration, min. *a*, 508; *b*, 943 (distance migrated about 46 cm.); *c*, 90; *d*, 206; *e*, 360; *f*, 1205 (distance migrated about 56 cm.). *g–j* bovine serum albumin, 1·15 g./100 ml., 9·3 V./cm.; *l*, *m*, human serum albumin from electrophoresis of serum diluted 1 : 4, 10·4 V./cm.; both albumins in phosphate, pH 8, $I = 0{\cdot}1$; times of migration, min.; *g*, 176; *h*, 778; *j*, 1142; *l*, 540; *m*, 293, after the freshly formed boundary had been left to diffuse for 12 hr. *k*, same bovine serum albumin as in *g–j* at about 1 g./100 ml., acetate, pH 4, $I = 0{\cdot}02$; distance migrated 8·1 cm. All patterns are from ascending boundaries, the direction of migration is towards the left.

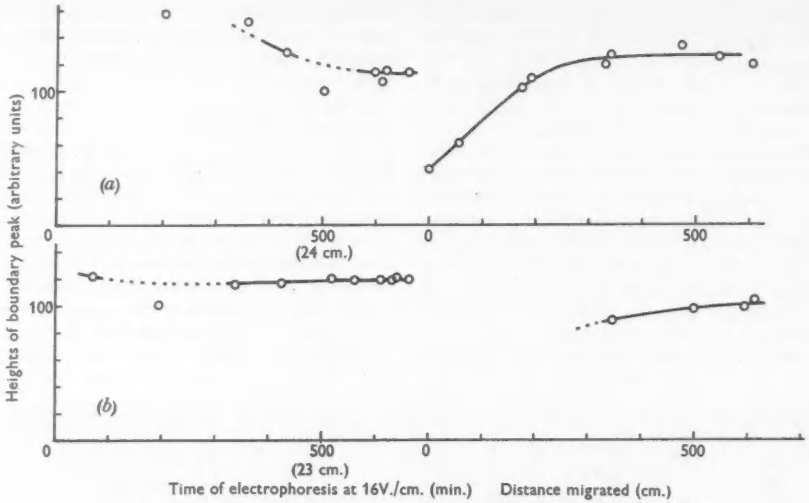

Fig. 3. Tests for electrophoretic homogeneity, HbO_2. For details see text.

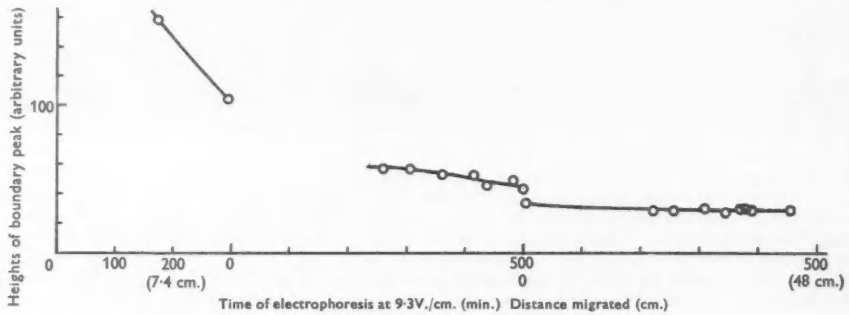

Fig. 4*a*.

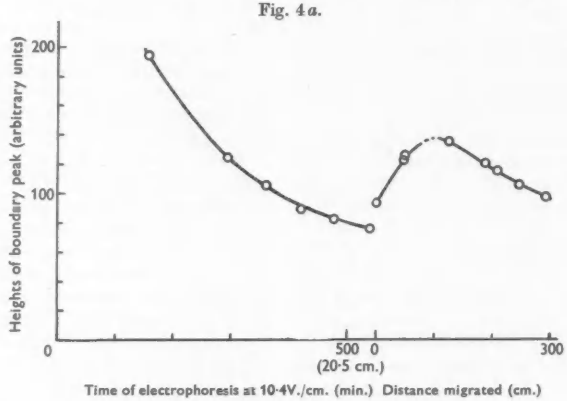

Fig. 4*b*.

Fig. 4. Tests for electrophoretic homogeneity, albumins. *a*, bovine serum albumin; *b*, human serum albumin from a patient with hypertension.

sharp during a migration over a distance considerably greater than the length of the U-tube, the slow broadening of the peak had to be interpreted as arising from heterogeneity of the preparation. Towards the end of the run the boundary had become very diffuse so that the relative changes in width due to heterogeneity were too small to produce noticeable changes in the height. The total distance migrated was 46 cm. Heterogeneity was confirmed in an electrophoresis experiment at pH 4 in which four major components were revealed (Fig. 2*k*).

measured in the absence of an electric current is used in the equations, a minimum value for the width can be derived. This was done in the case of oxyhaemoglobin. Duplicate runs gave 4·03 and $3{\cdot}95 \times 10^{-7}$ respectively. The oxyhaemoglobin concentrations in the adjusted solutions in Exps. 1 and 2, were $C_0 = 0{\cdot}9$ and 0·63 g./100 ml., $V_0 = 7{\cdot}3 \times 10^{-4}$ cm./sec. and K (for $C = 1{\cdot}15$) = 0·124 and 0·122 (duplicate experiments). With these values the curve of Fig. 1 had been computed. Fig. 5*a* shows a tracing of the boundary in Exp. 1 for comparison. The experi-

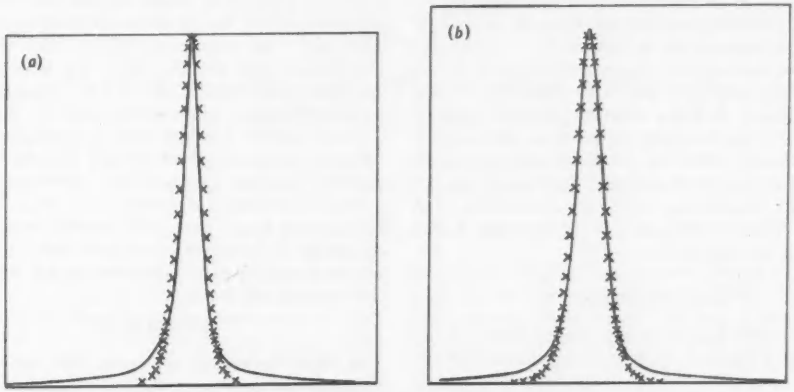

Fig. 5. Tracings of experimental boundary patterns with superimposed theoretical curves; *a*, HbO_2, after 433 min. migration; *b*, bovine albumin after 174 min. migration.

A similar kind of heterogeneity was observed with the albumin of a serum from a subject with hypertension (Fig. 2*l*, *m* and Fig. 4*b*). After the boundary had migrated for 22 cm. a new boundary was formed by the sharpening technique (Kahn & Polson, 1947) and left to diffuse for 12 hr. On renewed electrophoresis the peak became at first narrower and higher, but after 135 min. it began to broaden again. This broadening of the peak after an initial sharpening was a clear indication that no steady state of the boundary was possible with this material. Comparison of the widths of the boundaries in Fig. 2*h*, *l* shows that the bovine albumin was heterogeneous to a greater degree than the particular human sample. The albumin concentrations in the adjusted solutions were 1 g./100 ml. in both cases.

Calculation of the width of a boundary in the steady state

Haemoglobin. From equations (5) or (10) the width of the boundary peak at a given relative height can be calculated if V_0, K, C_0 and D' are known. It does not seem feasible to determine D' experimentally, but if the diffusion constant which can be

mental boundary peak was narrower near the top and broader near the base. The calculated minimum for the width of the boundary at half the maximum height ($0{\cdot}5\,h_{max.}$) then works out to $1{\cdot}9 \times 10^{-2}$ cm. The experimentally found widths of the peaks in the steady stage at $0{\cdot}5\,h_{max.}$ were 11 and 9×10^{-2} cm. respectively. These are about five times the calculated minimum values.

Albumin. The calculation of the minimum width of the peak at $0{\cdot}5\,h_{max.}$, using the values for the constants which apply to the conditions of the experiment with albumin, gave $3{\cdot}5 \times 10^{-2}$ cm. The widths of the boundary of bovine albumin found experimentally were 18 and 27×10^{-2} cm. after migrated distances of 6·6 and 11 cm. The potential gradient was 9·3 V./cm. The widths of the boundaries of serum albumin were 16·6, 24·5 and 28×10^{-2} cm. after the boundaries had migrated 6·4, 12·1 and 14·8 cm. The potential gradient was 10·4 V./cm. The degree of heterogeneity of the serum albumin was less than that of the bovine albumin. Fig. 5*b* shows a tracing of the boundary of Fig. 2*g* with a curve superimposed, calculated from equation (10) with a value of $\frac{V_0 K C_0}{D'}$ to give the best fit.

The tes for electrophoretic homogeneity

The following procedure may be suggested. A buffer is used in which the ascending boundary is sharper than the descending and in which the migration velocity is high at the maximum current permissible. A preliminary run is made, with the ascending side towards the open electrode vessel, the other being closed (Longsworth & MacInnes, 1939), until the ascending boundary has migrated 6 or 7 cm.. in order to ascertain that the material gives a single boundary only, or to separate slower impurities. The current is then interrupted, a fresh boundary is formed immediately below the original boundary and the boundary is left to diffuse. If, on restarting the current, the boundary becomes sharper, it is then observed for as long a period as possible, while a steady current of buffer solution is maintained in order to keep the boundary in position. Broadening of the boundary after an initial sharpening should be ascribed to electrophoretic heterogeneity. An accidental broadening due to mechanical disturbance will be followed by sharpening if the material is homogeneous.

DISCUSSION

The width which a boundary should reach in the steady state has been calculated assuming that the only factor which tends to cause spreading of the boundary is thermal diffusion of the protein ions. In the case of oxyhaemoglobin in which a steady state of a boundary has been realized experimentally, the width of the boundary was five times this value. This discrepancy is probably, in the first place, a consequence of the neglect of the diffusion of the buffer salt ions. Only a small part of the discrepancy between the width calculated and that found experimentally can be attributed to the effects of the presence of different ionic forms since, according to Ogston (1946), the mean life of the individual forms is likely to be of the order of 10^{-14} sec. and the spreading due to this cause small compared with that due to diffusion. On the other hand, the attainment of a steady state is strong evidence for the electrophoretic homogeneity of the main component of normal human red cells. Assumption of different interconvertible forms with comparatively long mean life times may contribute to the spreading. The reactions between these forms would have to be of non-ionic type.

The characteristic deviation of the experimental shape of the boundary pattern from that of the Gaussian error curve is similar to that of the calculated curve, but this deviation is much more pronounced, particularly near the base. The type of tailing-off observed here may be explained qualitatively by the tendency of the buffer salt ions to establish a linear concentration gradient across the protein boundary.

The conditions of pronounced boundary anomalies are not only useful in that they facilitate the formation of a steady state from a diffuse boundary, but they also provide a means of concentrating constituents, which are present in traces only, simultaneously with separating them from the main component. The less the difference between their mobilities and that of the main component, the greater the degree of concentration. By this means it was possible to isolate with comparatively little effort the components which had a slightly greater mobility than oxyhaemoglobin in sufficient quantity to study the spectral absorption. Drs E. M. Jope and E. Johnson found that this material was spectroscopically indistinguishable from the bulk of the oxyhaemoglobin as regards the visible region and the Soret band.*

SUMMARY

1. The theoretical shape of the pattern of a boundary in the steady state has been calculated.

2. A steady state, indicating electrophoretic homogeneity, has been realized experimentally with the main component of normal human red-cell extracts.

3. With crystallized bovine albumin, or with the albumin in a serum from a patient with hypertension, no steady state was attained. These materials were considered to be heterogeneous, although they showed a single peak during prolonged electrophoresis.

4. A method of testing for electrophoretic homogeneity has been outlined.

5. The presence in red-cell extracts of two electrophoretic components, not hitherto described, has been demonstrated.

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The Irreversibility of the Deamination of Threonine in the Rabbit and Rat

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It has been known for some time from perfusion and growth experiments with mammals that many amino-acids are deaminated to keto acids and that these can be reconverted to the original amino-acids. Thus, the fact that many essential amino-acids can be replaced in the diet by the D-enantiomorphs or by the corresponding hydroxy or keto acids indicates that deamination and reamination occur readily. However, the extent and the speed of such reactions was not fully realized until studies with isotopic nitrogen were initiated in the field of intermediary metabolism (for reviews see Schoenheimer, 1942; Schoenheimer & Rittenberg, 1940). It was shown that, after feeding to rats ammonium salts, glycine, L- or D-leucine or L-tyrosine labelled with ^{15}N, almost all the amino-acids isolated from the proteins of different tissues contained some excess ^{15}N. The amide nitrogen and the amino nitrogen of glutamic and aspartic acids always had the largest concentrations of the isotope, but some 'essential' amino-acids such as histidine and leucine also exchanged reversibly their α-amino nitrogen with that of other amino-acids or with ammonia. For other essential amino-acids such as tryptophan, methionine and phenylalanine, such a reversible deamination and reamination has not been demonstrated by the isotope method, but positive evidence for the occurrence of such reactions has been obtained from growth experiments using D-amino-acids and also related keto and hydroxy acids (for reviews, see Rose, 1938; Jackson & Chandler, 1939; Neuberger, 1948). Negative evidence from such nutritional experiments is not conclusive. Thus D-valine, D-leucine and D-isoleucine are not available for growth in the rat (Rose, 1938), and similar results have been reported for the mouse (Bauer & Berg, 1943). The chick can utilize D-leucine, but not D-isoleucine or D-valine (Grau & Peterson, 1946). On the other hand, Schoenheimer, Ratner & Rittenberg (1939), using L-leucine labelled with ^{15}N and deuterium, showed that this amino-acid exchanges its nitrogen with that of other amino-acids. Later, the inversion of D- to L-leucine in the rat was clearly demonstrated by the method of double labelling (Ratner, Schoenheimer & Rittenberg, 1940). These findings and the observation of Rose (1938) that the three keto acids corresponding to leucine, valine and isoleucine support growth indicate that these three essential aliphatic amino-acids yield nitrogen to, and accept nitrogen from, other amino-acids. The negative results of growth experiments with the D-amino-acids may be explained by assuming that the rate of deamination is too slow for the requirements of growth.

The only amino-acid which had been isolated by the Columbia workers and which never contained any excess ^{15}N was lysine (Foster, Schoenheimer & Rittenberg, 1939). It had been shown earlier (Foster, Rittenberg & Schoenheimer, 1938) that lysine, unlike other amino-acids, does not take up deuterium from the body water. By the method of double labelling (deuterium and ^{15}N) it was demonstrated that the products of deamination of lysine cannot be aminated again to form lysine (Weissman & Schoenheimer, 1941). This conclusion agrees fully with the observations that neither D-lysine (Berg, 1936) nor any known derivative of lysine, with the exception of ε-*N*-methyllysine and ε-*N*-acetyllysine

(Neuberger & Sanger, 1943, 1944), can replace L-lysine in the diet.

No evidence from isotope experiments was available to indicate whether the one remaining essential amino-acid, threonine, is reversibly deaminated in the mammalian organism. The finding of West & Carter (1938) that D-threonine is not available for growth in the rat could be taken to indicate that threonine resembles lysine in this respect. But as argued above, a rigorous conclusion cannot be drawn from such evidence. One of us (Elliott, 1949) has recently described a method which permits the isolation of threonine from relatively small quantities of protein. It was therefore decided to investigate whether threonine accepts nitrogen from other amino-acids. The first experiment was done with a rabbit, using a relatively small quantity of ^{15}N-containing glycine; the second experiment was carried out with a group of rats, using a relatively large amount of labelled amino-acid.

A preliminary communication on this work has been made by Elliott & Neuberger (1949).

EXPERIMENTAL

Rabbit experiment. Two male Hollingsworth half-lopped rabbits (1·61 and 1·65 kg. body wt.), which were being fed the Institute stock diet, were given glycine containing 31·69 atom percentage excess ^{15}N mixed with the food in divided doses over 4 days. The total amount of labelled glycine given was 0·4 g./kg. body wt. The animals were killed on the fifteenth day after the last administration of the isotopic compound. The muscles of the two animals were removed and minced. The protein was separated by the salting-out method of Deutsch, Eggleton & Eggleton (1938). The resulting mixed muscle protein was heated with several lots of water until the aqueous solution was free of SO_4^{--} and was then treated successively with warm ethanol and ether.

A portion (50 g.) of the air-dry protein was then hydrolysed and serine, threonine and glutamic acid were isolated as described by Elliott (1949); 1·34 g. of a mixture consisting of 56% threonine and 44% serine was obtained. This was recrystallized from 80% (v/v) aqueous ethanol and 120 mg. of the material was fractionated on a starch column giving 65 mg. of L-threonine and 47 mg. of DL-serine. Both samples were recrystallized for analysis. The threonine gave a titration figure of 99·4% of the theoretical value (average of three analyses) by the periodate method (Rees, 1946), whilst the serine gave 96·4% (average of three analyses). The glutamic acid hydrochloride was recrystallized from aqueous HCl and the free amino-acid was isolated by exact neutralization of the hydrochloride and recrystallized from water–ethanol. Glutamic acid had m.p. 201° (uncorr.); $[\alpha]_D^{20°}$ +31·5° (*c*, 2·0 in 5·5N-HCl). (Found: N, 9·4. Calc. for $C_5H_9O_4N$: N, 9·5%.) Tyrosine was obtained from the neutral amino-acid fraction and recrystallized three times from water. It had $[\alpha]_D^{21°}$ −7·9° (*c*, 1·5 in 5·5N-HCl). (Found: N, 7·5. Calc. for $C_9H_{11}O_3N$: N, 7·7%.)

Another portion of the air-dry protein (50 g.) was hydrolysed by refluxing with 300 ml. of 5·5N-HCl for 12 hr. Excess HCl was removed, the basic amino-acids were precipitated with phosphotungstic acid and most of the glutamic acid was obtained as the hydrochloride. The acidic solution was concentrated to dryness and the glycine was obtained as the trioxalatochromiate complex (Bergmann & Niemann, 1938). The complex was decomposed and the glycine benzoylated. The hippuric acid (1·32 g.) was crystallized and recrystallized from water; it had m.p. (uncorr.) 189°. (Found: N, 7·7; Calc. for $C_9H_9O_3N$: N, 7·8%.)

Rat experiment. One male and two female rats of body wt. 250, 235 and 215 g., respectively, were kept on the following diet which was fed *ad lib.* for 18 days before isotopic glycine was given: salt mixture (U.S. Pharmacopoeia 1947, p. 721, no. 2), 4%; yeast powder, 5%; casein, 3%; maize starch, 80%; arachis oil, 6·4% and cod liver oil, 1·6%. The protein content of the diet was about 5·5%. At the end of the preliminary period the rats had lost 10, 3 and 12 g. respectively. The rats then received on three successive days 150 mg. of isotopic glycine/rat/day mixed with the food. The glycine had 31·5 atom percentage excess ^{15}N. The rats were killed 24 hr. after the last administration of the labelled glycine. Liver, spleen, kidney, pancreas, heart and the washed small intestine of the three rats were combined and minced. The product was heated with 50% (v/v) ethanol and the resulting precipitate was repeatedly washed with the same solvent. Fat was removed by successive treatment with ethanol, acetone and ether. 10·45 g. of air-dry material was obtained.

The protein was hydrolysed and the basic amino-acids were removed with phosphotungstic acid. Glutamic acid was isolated as the hydrochloride; this fraction was unfortunately lost. Tyrosine was obtained from the neutral amino-acid fraction; the recrystallized material had $[\alpha]_D^{18°}$ −7·8° (*c*, 1·5 in 5·5N-HCl). (Found: N, 7·6. Calc. for $C_9H_{11}O_3N$: N, 7·7%.) From the basic fraction arginine was obtained as monoflavianate; this was converted with $Ba(OH)_2$ to the monohydrochloride, $[\alpha]_D^{20°}$ +26·1° (in terms of free amino-acid) (*c*, 1·4 in 5·5N-HCl). (Found: N, 26·4. Calc. for $C_6H_{15}O_2N_4Cl$: N, 26·6%.) Histidine was removed from the mother liquor obtained in the precipitation of arginine by Ag^+ ions and the lysine reprecipitated as phosphotungstate after removal of NH_3 by aeration. The lysine phosphotungstate was converted to the picrate which was recrystallized, m.p. (decomp.) 265°. The picrate was decomposed with HCl and the lysine crystallized as monohydrochloride which was recrystallized from aqueous ethanol, $[\alpha]_D^{20°}$ +25·5° (*c*, 1·32 in 5·5N-HCl) (in terms of the free amino-acid). (Found: N, 7·6. Calc. for $C_6H_{15}O_2N_2Cl$: N, 7·6%.)

The serine threonine mixture, which was obtained as described by Elliott (1949) and amounted to 170 mg., was recrystallized. By periodate titration (Rees, 1946) it was found to contain 73·7% threonine and 24·5% serine. The recrystallized material (100 mg.) was placed on the starch column. Yield of threonine was 46 mg., which was recrystallized for analysis. This product titrated to 100% of the theoretical value by the periodate method (average of three analyses). The column, after most of the threonine had passed through, blocked up and serine could not be isolated. Isotope analyses were therefore done on the serine threonine mixture and the values of serine were calculated after allowing for the threonine present.

In this experiment glycine was obtained from that fraction of the hydrolysate which originally contained oxazolines derived from serine and threonine, and benzimino-ethyl ether derivatives mainly of glycine, alanine and valine. As shown by Elliott (1949), alkali converts these latter into the corresponding benzamido acids, whilst the two oxazolines

are stable to the alkali treatment used, but are converted by the subsequent acidification to *O*-benzoyl amino-acids which, unlike the *N*-benzoyl compounds, are not extracted into organic solvents from the acidified aqueous solution. The mixture of *N*-benzoyl compounds from the first ethyl acetate extract was hydrolysed by refluxing with 5·5 N-HCl for 8 hr. Benzoic acid was removed by filtration and extraction with ether. The aqueous solution was concentrated to dryness, and the residue taken up in water (10 ml.). K oxalatochromiate (4·5 g.), ethanol (17·5 ml.) and 10% (w/v) HCl (2·5 ml.) were then added. The mixture was shaken for 6 hr., filtered, and the precipitate washed with 12·5 ml. of 75% (v/v) ethanol. The precipitate was dissolved in water (9 ml.) to which 1·3 N-NaOH (7·5 ml.) had been added. Benzoylation was carried out in the usual manner using 1·87 ml. of benzoyl chloride and 10 ml. of 1·3 N-NaOH. After acidification excess benzoic acid was extracted with benzene and hippuric acid with ethyl acetate. The ethyl acetate solution was dried and concentrated to dryness. The residue was crystallized and recrystallized from water. The hippuric acid had m.p. 190° (uncorr.). (Found: N, 7·6. Calc. for $C_9H_9O_2N$: N, 7·8%.)

RESULTS

Rabbit experiment (Table 1). In this experiment the amount of isotopic glycine fed was relatively small. It is not surprising, therefore, that the concentration of ^{15}N even in glycine was rather low.

Table 1. *Atom percentage excess ^{15}N of amino-acids isolated from the muscle proteins of two rabbits fed isotopic glycine*

Amino-acid	Atom percentage excess ^{15}N
Glycine	0·092
Serine	0·086
Glutamic acid	0·024
Threonine	0·000
Tyrosine	0·014

Serine had almost the same value for atom percentage excess ^{15}N as glycine. The corresponding figures for glutamic acid and tyrosine were considerably lower, but well outside the limits of error of the mass spectrometric analysis which are about ±0·005%. Threonine contained no excess ^{15}N.

Rat experiment (Table 2). In this experiment the amount of isotopic glycine administered/kg. body weight was about four times larger than in the rabbit

Table 2. *Atom percentage excess ^{15}N of amino-acids isolated from the proteins of the internal organs of three rats fed isotopic glycine*

Amino-acid	Atom percentage excess ^{15}N
Glycine	3·500
Serine	3·300
Threonine	0·000
Arginine	0·366
Tyrosine	0·107
Lysine	0·000

experiment. Moreover, it was to be expected, on the basis of the results of Shemin & Rittenberg (1944), that the concentration of the isotope in the protein of the internal organs immediately after the administration of the labelled compound would be much higher than the corresponding values for muscle more than 2 weeks after the beginning of the experiment. The ^{15}N values were, in fact, about 10–30 times higher than in the first experiment. Glycine and serine contained very high, and again very similar, amounts of ^{15}N. Both arginine and tyrosine appeared to have exchanged their nitrogen with that of glycine, whilst lysine and threonine showed no excess of ^{15}N.

DISCUSSION

The results show clearly that, in the rat, threonine isolated from tissue proteins after feeding ^{15}N-labelled glycine does not contain any excess ^{15}N, whilst all other amino-acids isolated, except lysine, contain relatively large amounts of the labelled nitrogen. If a nitrogen-free derivative of threonine can be reaminated at all to form threonine, the speed of the reaction must be exceedingly slow, but this possibility cannot be completely excluded. It appears more probable that deamination of threonine, like that of lysine, is irreversible in the rat and that either threonine is not converted into the corresponding α-keto acid or the keto acid is not reaminated. The fact that the L-amino-acid oxidases of rat kidney and liver do not attack L-threonine (Blanchard, Green, Nocito & Ratner, 1944) may indicate that α-keto-β-hydroxybutyric acid is not a metabolite of threonine in the rat. With the rabbit the evidence is less conclusive, since the amount of isotopic glycine fed was smaller; but the data suggest that in the rabbit too, deamination of threonine is not reversible. It thus appears that threonine and lysine differ from other essential amino-acids, which, like the non-essential amino-acids, take part in a reversible exchange of α-nitrogen.

Another interesting point emerges from this work. Shemin (1946) has conclusively demonstrated that serine is converted to glycine in the rat and guinea pig. The converse reaction, transformation of glycine to serine, has also been shown to occur in whole rats (Sakami, 1948; Goldsworthy, Winnick & Greenberg, 1949), rat-liver slices (Siekevitz, Winnick & Greenberg, 1949) and rat-liver homogenates (Winnick, Moring-Claesson & Greenberg, 1948). In all these experiments ^{14}C and carrier serine were used. Our results show that in this conversion of glycine to serine, most or all of the nitrogen is retained. It thus appears that the reversible interconversion of glycine and serine is rapid compared with other metabolically reversible reactions in which the two amino-acids participate. One may also conclude that with both compounds oxidative deamination to the keto acid followed by reamination is slow. In fact, reamination of the keto acid may not occur at

all and the ^{15}N found in glycine after feeding ^{15}N-labelled amino-acids other than glycine and serine, may, as already suggested by Shemin (1946), arise by synthesis of glycine from a substance other than glyoxylic acid.

SUMMARY

1. Glycine containing excess ^{15}N was fed to rabbits and rats, and various amino-acids were isolated from protein of muscle and internal organs respectively. The glycine, serine, glutamic acid, arginine and tyrosine contained varying concentrations of isotopic nitrogen. Lysine and threonine had no excess ^{15}N.

2. It is concluded that threonine, like lysine, does not take part in the reversible transfer of nitrogen which is observed with other amino-acids, both 'essential' and 'non-essential'.

3. The glycine and serine isolated contained similar proportions of ^{15}N. This is taken to indicate that the conversion of glycine to serine is very fast compared with other reversible reactions in which the two amino-acids participate.

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The Oxidation of Various Synthetic α-Amino-acids by Mammalian D-Amino-acid Oxidase, L-Amino-acid Oxidase of Cobra Venom and the L- and D-Amino-acid Oxidases of *Neurospora crassa*

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Through the courtesy of Sir Charles Harington, F.R.S., National Institute for Medical Research, Hampstead (Elliott, Fuller & Harington, 1948; Elliott & Harington, 1949) and of Dr B. A. Hems, Glaxo Laboratories Ltd. (Elks, Hems & Ryman, 1948) we obtained twenty-seven synthetic α-mono-amino-acids not commonly available. The behaviour of these substances towards D-amino-acid oxidase of sheep kidney, L-amino-acid oxidase of cobra venom and the L- and D-amino-acid oxidase of *Neurospora crassa* has been investigated. The rates of oxidation of the common amino-acids already examined by previous authors were also measured in order to obtain series of directly comparable data.

EXPERIMENTAL

Substrates

All amino-acids used were DL- unless otherwise stated. The following special DL-amino-acids were examined.

Straight chain α-amino-monocarboxylic acids. α-Aminocaprylic, α-aminohendecanoic, α-aminolauric, α-aminostearic acids.

Aliphatic α-aminodicarboxylic acids. α-Aminoadipic, 1-aminohendecane-1:11-dicarboxylic, α-aminopimelic, α-amino-β-*n*-octylsuccinic, α-amino-β-*n*-butylsuccinic, α-amino-α-*n*-propylsuccinic acids.

α-Amino-acids with no hydrogen atoms attached to the α-carbon. α-Amino-α-methyltridecanoic, α-amino-α-methylcaprylic, α-amino-α-methylvaleric, α-amino-α-methylhendecanoic, α-amino-α-*n*-heptylpelargonic, α-amino-α-*n*-amyloenanthic, α-amino-α-ethylbutyric acids.

α-Amino-acids with a cyclic substituent attached to the β-carbon. *p*-Amino-phenylalanine, *p*-dimethylaminophenylalanine, β-quinolyl(4)alanine, β-quinolyl(2)alanine, *p*-(β-aminoethyl)phenylalanine,* β-pyridyl(4)alanine, β-6-methoxyquinolyl(4)alanine, *p*-guanidinomethylphenylalanine, β-furyl(2)alanine,* ε-*N*-sulphanilyllysine.*

α-Amino-acids having no hydrogen atom attached to the α-carbon were not oxidized by any of the enzymes tested, as was to be expected (Wieland & Bergel, 1924). Of the dicarboxylic amino-acids those having branched chains (β-substituted aspartic acids) were not attacked. In view of the negative results obtained with these substances these amino-acids are not listed in the following tables.

EXPERIMENTS WITH D-AMINO-ACID OXIDASE OF SHEEP KIDNEY

Procedure

A crude extract of acetone-dried sheep kidney cortex (Krebs, 1935) was prepared immediately before use by shaking 1 g. of dried kidney for 5 min. with 20 ml. of 0·1 M-pyrophosphate buffer, pH 8·4, and filtering through an agar filter paper. The rate of oxidation was measured manometrically in Warburg flasks of about 23 ml. capacity provided with centre well and side arm. The quantity of enzyme solution used varied from compound to compound and is stated in Table 1. It was so adjusted as to give pressure changes not exceeding 20 mm. in 5 min. Control experiments showed that under the conditions used the rate of oxidation was directly proportional to the quantity of enzyme solution used. The main compartment of the Warburg flasks contained the enzyme solution and sufficient 0·1 M-pyrophosphate buffer to bring the volume to 4 ml. The buffer was prepared by adding 1·6 ml. of N-HCl to 20 ml. of 0·2 M-sodium pyrophosphate and diluting to 40 ml. (Warburg & Christian, 1938). Of the DL-amino-acids $4·5 \times 10^{-5}$ g.mol. were weighed into the side arm; the powder was moistened with 0·5 ml. of water or, in the case of acidic substances, with 0·5 ml. of NaOH solution of sufficient strength to neutralize the acid. This procedure was adopted because the limited solubility made the use of solutions impracticable in many cases. The gas space of the flasks contained O_2. The bath temperature was 40°. The contents of the side arm and main compartment were mixed after equilibration and readings were taken at 5 min. intervals. Undissolved material was present at the end of the experiments with α-aminocaproic, α-aminohendecanoic, α-aminolauric, α-aminostearic, α-amino-α-methyltridecanoic, α-amino-α-methylhendecanoic, α-amino-α-*n*-heptylpelargonic, α-amino-α-*n*-amyloenanthic acids, and with cystine.

Differences in the potency of enzyme preparations from different kidneys

Wide variations were observed in the potency of the enzyme solutions prepared from different sheep kidneys. For the test of activity 1 ml. of extract was used and DL-alanine served as substrate. The conditions were as described in the preceding paragraph. The O_2 absorption for the first 5 min. varied between 6 and 145 μl. No correlation was found between D-amino-acid oxidase activity and breed, age or sex of the animals, but the limited information so far available suggests a seasonal factor. Eight kidneys tested between April and June all showed low activity (13–22 μl. of O_2 absorbed in 5 min.). Of sixteen specimens collected between August and October seven gave very high values (107–145 μl. of O_2 in 5 min.); four fairly active (78–90 μl. of O_2 in 5 min.); four were moderately active (25–45 μl. of O_2 in 5 min.) and only one had an activity below 25 μl. of O_2 in 5 min. For the following experiments an acetone powder of high potency was used. The cause of the variations in potency is being further investigated.

Results

Rate of oxidation of various amino-acids

The data obtained are shown in Table 1. All available amino-acids were examined with the same enzyme solution which contained 2·025% dry matter (excluding buffer) and which gave a blank oxygen absorption of 3 μl./ml. in 5 min. and 4·5 μl./ml. in 10 min. The figure in the last column (q_{O_2} = μl. of O_2 absorbed/mg. dried kidney in enzyme solution/hr.) is calculated from the oxygen-uptake values for 10 min. and the dry matter (excluding buffer) of the enzyme solution. It represents a relative measure of the rate of oxidation of various amino-acids.

It will be noted that in most cases the 5 min. value for the oxygen uptake was less than half of the 10 min. value. The slow initial rate was probably due to a true lag period, the cause of which is not clear. In a few cases, for example, that of phenylalanine, the rate of reaction decreased during the second 5 min. period.

Straight-chain α-aminomonocarboxylic acids. The data in Table 1 show that the rates of oxidation of the C_4 and C_6 compounds were about half that of alanine. The C_5 compound reacted more slowly than the C_4 and C_6 compounds, and the C_8 compound was very slowly oxidized. The higher homologues did not react.

Branched-chain aliphatic α-aminomonocarboxylic acids. Valine was oxidized rather faster than its

* Synthesized in the laboratory of Sir Charles Harington (unpublished).

Table 1. *Oxidation of amino-acids by* D-*amino-acid oxidase of sheep kidney*

(For conditions see text. All data are corrected for the blank O_2 absorption of the enzyme solution. q_{O_2} = (μl. of O_2 absorbed)/(mg. of dried kidney in enzyme solution × hr.). q_{O_2} was calculated from the O_2 uptake during the first 10 min.)

Amino-acid	Volume of enzyme solution (ml.)	O_2 absorbed (μl.) after 5 min.	10 min.	20 min.	30 min.	q_{O_2}
Straight chain α-aminomonocarboxylic acids:						
Alanine	0·2	20	43	93	132	64
α-Aminobutyric acid	0·5	29	53	103	135	31
α-Aminovaleric acid	0·5	15	34	74	106	20
α-Aminocaproic acid	0·3	15	37	74	109	36
α-Aminocaprylic acid	4·0	18	26	48	56	1·9
α-Aminohendecanoic acid	4·0	Nil	Nil	Nil	Nil	Nil
α-Aminolauric acid	4·0	Nil	Nil	Nil	Nil	Nil
α-Aminostearic acid	4·0	Nil	Nil	Nil	Nil	Nil
Branched chain aliphatic α-aminomonocarboxylic acids:						
Valine	0·5	27	60	127	180	35
Leucine	1·0	20	47	92	127	13·9
Isoleucine	0·5	21	37	71	101	22
Aliphatic α-aminodicarboxylic acids:						
Aspartic acid	4·0	6	19	38	57	1·4
Glutamic acid	4·0	Nil	Nil	Nil	Nil	Nil
α-Aminoadipic acid	4·0	Nil	Nil	Nil	Nil	Nil
α-Aminopimelic acid	4·0	Nil	Nil	Nil	Nil	Nil
1-Aminohendecane-1:11-dicarboxylic acid	0·5	32	70	126	162	41
α-Amino-acids with cyclic substituents:						
Phenylalanine	0·5	29	44	67	74	26
Tyrosine	0·1	37	64	110	137	190
p-Aminophenylalanine	0·2	20	44	82	112	65
p-Dimethylaminophenylalanine	0·3	12	33	106	170	32
p-(β-Aminoethyl)phenylalanine	1·0	25	55	96	130	16
p-Guanidinomethylphenylalanine	0·5	28	56	102	135	33
β-Quinolyl(4)alanine	1·0	12	28	60	82	8·3
β-Quinolyl(2)alanine	0·1	10	20	32	44	59
β-6-Methoxyquinolyl(4)alanine	0·2	5	18	42	57	27
β-Pyridyl(4)alanine	0·2	36	64	111	139	95
β-Furyl(2)alanine	1·0	18	40	107	—	11·9
ε-*N*-Sulphanilyl-lysine	4·0	Nil	Nil	Nil	Nil	Nil
Histidine	1·0	13	21	38	47	6·2
Tryptophan	0·2	8	25	52	80	37
Diaminomonocarboxylic acids:						
Lysine	4·0	2	8	12	19	0·6
Ornithine	2·0	10	21	43	56	3·1
Unclassified α-amino-acids:						
Serine	0·5	32	71	127	171	42
Threonine	4·0	11	28	55	76	2·1
Proline	0·1	24	50	90	115	148
Piperidine-α-carboxylic acid	2·0	7	18	22	30	2·6
Methionine	0·2	24	54	107	146	80
Cystine	2·0	6	13	26	37	1·9

straight-chain isomer. In the case of the aminocaproic acids on the other hand, the two branched-chain isomers, leucine and isoleucine, were oxidized at lower rates than the straight-chain isomer.

Aliphatic α-aminodicarboxylic acids. Aspartic acid was very slowly oxidized, whilst glutamic acid, aminoadipic and aminopimelic acids were not attacked. The homologous C_{13} compound, in contrast, was oxidized at a high rate.

Amino-acids with cyclic substituents. Tyrosine showed the highest rate of oxidation of all the substrates tested. The substitution of the *p*-hydroxyl group of tyrosine by an amino group considerably reduced the rate of oxidation. Still lower rates were obtained with *p*-dimethyl-aminophenylalanine, *p*-(β-aminoethyl)phenylalanine, and *p*-guanidinomethylphenylalanine. β-Quinolyl(4)alanine reacted slowly in contrast to β-quinolyl(2)alanine which gave

a high rate; the rate of oxidation of β-6-methoxyquinolyl(4)alanine was between these two values. β-Pyridyl(4)alanine was oxidized rapidly and β-furyl(2)alanine slowly, but ε-*N*-sulphanilyllysine was not attacked. Tryptophan was oxidized at about half the rate of alanine and histidine was only slowly attacked. The products of oxidation of β-quinolyl-(4)alanine, β-quinolyl(2)alanine and of β-6-methoxyquinolyl(4)alanine formed bright yellow solutions.

Total oxygen uptake

The total oxygen uptake did not exceed the theoretical value of one molecule of oxygen for four molecules of DL-amino-acid, except in the case of β-*p*-dimethylaminophenylalanine. This substance, on addition to kidney extract, absorbed four times the amount of oxygen expected, namely one molecule of oxygen per molecule of the DL-form.

For example, $2{\cdot}25 \times 10^{-5}$ g.mol. of the DL-amino-acid absorbed 503 μl. ($2{\cdot}25 \times 10^{-5}$ g.mol.) of O_2 in 270 min. The greater part of this (410 μl., equiv. to 3·25 atoms of O_2) was absorbed in the first 90 min. The amount of O_2 consumed in successive 30 min. periods was 183, 167, 60, 25, 20, 15, 10 and 10 μl. respectively. The amount of NH_3 produced during the incubation was equivalent to 0·5 molecule/molecule of DL-compound added. E.g. 9·35 mg. of substrate yielded 0·355 mg. of NH_3. In an experiment where the reaction was stopped when one atom of oxygen had been absorbed for each molecule of substrate the formation of NH_3 had already reached the maximum value. These yields are in accordance with the assumption that the D-form only is deaminated.

No carbon dioxide was produced from *p*-dimethylaminophenylalanine. The reaction product was soluble and brown coloured. It has not been identified.

Neither tyrosine nor phenylalanine, nor *p*-aminophenylalanine absorbed more than the expected amount of oxygen under the same conditions. Dimethylaniline, *p*-dimethylaminoazobenzene and *p*-dimethylaminobenzaldehyde were not attacked, but *p*-dimethylaminophenylacetate was rapidly oxidized. This substance is autoxidizable in pyrophosphate buffer, pH 8·4, in the absence of kidney extract, but the rate of oxidation is much increased by addition of 'D-amino-acid oxidase'. Boiled enzyme has no effect. This phenomenon is under further investigation.

Oxidation of mixtures of amino-acids. It was thought that amino-acids which are not oxidized by the enzyme might, nevertheless, combine with it and inhibit the oxidation of other amino-acids. However, no inhibitions were observed when α-amino-α-ethylbutyric, α-amino-β-*n*-octylsuccinic, α-aminopimelic or α-aminoadipic acids were added together with alanine. Nor had α-aminopimelic acid any effect on the rate of oxidation of 1-aminohendecane-1:11-dicarboxylic acid.

EXPERIMENTS WITH L-AMINO-ACID OXIDASE OF COBRA VENOM

Procedure

The source of this enzyme was dried cobra venom kindly provided by Dr C. H. Kellaway, F.R.S. The conditions of the test were similar to those used by Zeller & Maritz (1945). The main compartment of the Warburg cup contained 1 mg. of cobra venom freshly dissolved in 4 ml. of 0·067M-phosphate buffer, pH 7·2, and 1 drop of octanol. To decompose the H_2O_2 formed during the oxidation, 0·1 ml. of catalase in the form of a liver homogenate (1 part rat liver : 10 parts water) was added. The substrate ($4{\cdot}5 \times 10^{-5}$ g.mol. of the DL-compound except in the case of arginine when half this amount of the L-compound was used) was weighed into the side arm and moistened with 0·5 ml. of water or the equivalent amount of NaOH; 2N-NaOH (0·2 ml.) was placed in the centre well. The gas space contained O_2. The temperature was 40°. On addition of 0·5 ml. of water from the side arm to the enzyme solution a positive pressure of about 4 mm. was observed, and all the readings were corrected accordingly. No explanation can be offered for this effect.

Results

Rate of oxidation of various amino-acids

The results of the measurements are shown in Table 2. As the cup contained 1 mg. of dry enzyme, the 60 min. value is comparable to the q_{O_2} value given in the last vertical column of Table 1.

Straight chain α-aminomonocarboxylic acids. The C_3, C_4, C_{11}, C_{12} and C_{18} compounds did not react, whilst the C_5, C_6 and C_8 compounds were oxidized. The highest rate was shown by the C_6 compound.

Branched-chain aliphatic α-aminomonocarboxylic acids. The only member of this group to react was leucine, the rate of oxidation being similar to that of its straight-chain isomer.

Aliphatic α-aminodicarboxylic acids. Of the five α-amino-acids of the group tested only the C_{11} compound was attacked, and the rate of oxidation was slow. The C_4, C_5, C_6 and C_7 homologues were not oxidized.

Amino-acids with cyclic substituents. Tyrosine gave the highest rate of oxidation and phenylalanine the second highest. Of the twelve other substances tested ten were oxidized. Among the two substances which did not react was histidine. The oxidation product of β-quinolyl(4)alanine, β-quinolyl(2)alanine and of β-6-methoxyquinolyl(4)alanine showed the same bright yellow colour noted in the experiments with D-amino-acid oxidase.

Diaminomonocarboxylic acids. Ornithine and lysine were not attacked.

Unclassified amino-acids. Methionine was slowly oxidized, but serine, threonine, proline and cystine did not react.

Total oxygen uptake

In some cases the rate of reaction was too slow to reach the theoretical value of 252 μl. of oxygen

Table 2. *Oxidation of amino-acids by* L-*amino-acid oxidase of cobra venom*

(For conditions see text. All data corrected for initial gas output of 6 μl.)

Amino-acid	O_2 absorbed (μl.) after 10 min.	20 min.	30 min.	1 hr.	2 hr.	4 hr.	5 hr.	6 hr.	22 hr.
Straight-chain α-aminomonocarboxylic acids:									
Alanine; α-aminobutyric acid; α-aminohendecanoic acid; α-aminolauric acid and α-aminostearic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
α-Aminovaleric acid	6	12	18	31	51	92	—	125	239
α-Aminocaproic acid	21	33	44	74	117	192	—	227	261
α-Aminocaprylic acid	7	17	25	50	81	140	—	176	213
Branched-chain aliphatic α-aminomonocarboxylic acids:									
Valine; isoleucine	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Leucine	14	28	41	77	132	203	—	134	256
Aliphatic α-aminodicarboxylic acids:									
Aspartic acid; glutamic acid; α-aminoadipic acid; α-aminopimelic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
1-Aminohendecane-1:11-dicarboxylic acid	—	6	9	18	41	76	—	104	168
α-Amino-acids with cylic substituents:									
Histidine; ε-*N*-sulphanilyllysine	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Phenylalanine	19	39	51	111	204	274	—	—	—
Tyrosine	35	86	136	246	306	346	—	388	477
p-Aminophenylalanine	13	29	51	113	256	489	—	582	766
p-Dimethylaminophenylalanine	5	17	32	86	189	338	372	—	—
p-(β-Aminoethyl)phenylalanine	—	—	—	12	22	44	—	60	149
p-Guanidinomethylphenylalanine	—	11	14	30	50	89	99	—	—
β-Quinolyl(4)alanine	—	20	27	47	83	129	—	169	236
β-Quinolyl(2)alanine	—	—	9	15	23	37	—	51	—
β-6-Methoxyquinolyl(4)alanine	—	8	13	28	50	84	—	106	149
β-Pyridyl(4)alanine	7	16	26	58	108	184	—	230	282
β-Furyl(2)alanine	12	30	45	85	152	248	271	—	—
Tryptophan	10	26	38	76	156	266	—	356	—
Diaminomonocarboxylic acids:									
Ornithine; lysine	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Unclassified α-amino acids:									
Serine; threonine; proline; piperidine-α-carboxylic acid; cystine	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Methionine	18	36	52	94	156	230	—	272	313
Arginine	7	12	14	23	38	—	—	—	—

within the time of the experiment. In the others the final value was of the expected order except in four cases where the oxygen uptake exceeded the theoretical figure. In the experiment recorded in Table 2 tyrosine absorbed 1·9, tryptophan 1·4, and *p*-aminophenylalanine 3·05 atoms of oxygen. In the case of β-furyl(2)alanine and of *p*-dimethylaminophenylalanine the oxygen uptake had not stopped at the end of the experiment, and to measure the final value for these substances further experiments were set up with larger quantities of enzyme (4 mg.) and smaller quantities of substrate ($2{\cdot}25 \times 10^{-5}$ g.mol.). Under these conditions β-furyl(2)alanine absorbed 2·2 atoms of oxygen and *p*-dimethylaminophenylalanine 3·5 atoms. The solutions in which *p*-dimethylaminophenylalanine and *p*-aminophenylalanine had undergone oxidation were dark brown.

Experiments with the L-amino-acid oxidase of *Neurospora crassa*

Procedure

It was found in the course of the study, as already reported elsewhere (Bender, Krebs & Horowitz, 1949), that some strains of *N. crassa* produce an L-amino-acid oxidase, others a D-amino-acid oxidase. The experiments were carried out with the strain 3411 of the National Collection of Type Cultures. It was grown on a medium similar to that used by Horowitz & Beadle (1943), which contained (w/v) 0·5% ammonium tartrate, 0·1% NH_4NO_3, 0·1% KH_2PO_4, 0·01% NaCl, 0·05% $MgSO_4.7H_2O$, 0·01% $CaCl_2$, 2% sucrose, 0·3% (dry) malt extract and 0·01% (dry) yeast extract. In addition, the following quantities of trace elements were added per litre: $Na_2B_4O_7$, 0·05 mg.; Na_2MoO_4, 0·04 mg.; $FeSO_4.7H_2O$, 1 mg.; $CuSO_4.5H_2O$, 0·4 mg.; $MnSO_4.7H_2O$, 0·1 mg. and $ZnSO_4.7H_2O$, 9 mg. The medium (150 ml.) was filled into 700 ml. conical Roux bottles, and autoclaved at

15 lb. pressure for 15 min. At an early stage of the work, the organism lost its ability to sporulate; it was propagated by transferring small pieces of mycelium to the new medium.

The cultures were incubated at 25° for 14–18 days. This period was found to give the maximum enzymic activity. The yield of mycelium per bottle averaged 3·5–4·0 g. wet wt. The harvested mycelia were briefly rinsed with distilled water, squeezed dry through muslin, and disintegrated in a Waring blender with 2 ml. of 0·0167M-pyrophosphate buffer pH 8·4 per g. wet wt. of mycelium. The material was centrifuged and the supernatant, after dilution with 0·25 vol. of 0·2M-pyrophosphate buffer, pH 8·4, was used as a source of the oxidase.

The O_2 uptake on addition of the amino-acids was measured in Warburg flasks at 30°. The main compartment contained 2 ml. of the mycelium extract and 2 ml. of 0·1M-pyrophosphate buffer, pH 8·4. 2N-NaOH (0·2 ml.) was used in the inner cup; the gas space contained O_2. Of the DL-amino-acids $4·5 \times 10^{-5}$ g.mol. were weighed into the side arm and moistened with 0·5 ml. of 0·1M-pyrophosphate buffer, or, in the case of acidic substrates, with 0·5 ml. of NaOH solution of sufficient strength to neutralize the acid.

Table 3. *Oxidation of amino-acids by the* L-*amino-acid oxidase of* Neurospora crassa (*Strain* 3411 *N.C.T.C.*)

(For conditions and the calculation of q_{O_2} see text. All data corrected for the blank O_2 absorption of the enzyme solution.)

Amino-acid	O_2 absorbed (μl.) after 5 min.	10 min.	20 min.	30 min.	60 min.	120 min.	180 min.	q_{O_2}
Straight-chain α-aminomonocarboxylic acids:								
Alanine	26	65	144	216	—	—	—	41
α-Aminobutyric acid	52	113	238	—	—	—	—	64
α-Aminovaleric acid	26	97	228	—	—	—	—	75
α-Aminocaproic acid	16	73	182	232	—	—	—	60
α-Aminocaprylic acid	15	31	52	78	104	124	139	17
α-Aminohendecanoic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
α-Aminolauric acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
α-Aminostearic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Branched-chain aliphatic α-aminomonocarboxylic acids:								
Valine	5	11	22	42	72	165	—	6
Leucine	28	102	223	—	—	—	—	78
Isoleucine	14	45	91	135	191	213	—	33
Aliphatic α-aminodicarboxylic acids:								
Aspartic acid	—	4	9	14	22	30	31	5
Glutamic acid	1	10	22	45	81	135	176	9
α-Aminoadipic acid	25	83	160	213	251	—	—	61
α-Aminopimelic acid	48	108	214	—	—	—	—	63
1-Aminohendecane-1:11-dicarboxylic acid	2	21	52	83	244	—	—	20
α-Amino-acids with cyclic substituents:								
Phenylalanine	21	70	168	276	—	—	—	41
Tyrosine	—	20	85	168	220	448	672	24
p-Aminophenylalanine	14	42	104	193	293	341	390	29
p-Dimethylaminophenylalanine	26	80	181	313	464	580	612	57
p-(β-Aminoethyl)phenylalanine	1	5	35	61	140	—	—	16
p-Guanidinomethylphenylalanine	2	19	45	83	224	—	—	18
β-Quinolyl(4)alanine	50	94	220	255	—	—	—	66
β-Quinolyl(2)alanine	43	100	200	214	—	—	—	60
β-6-Methoxyquinolyl(4)alanine	21	70	190	—	—	—	—	41
β-Pyridyl(4)alanine	43	95	212	247	—	—	—	55
β-Furyl(2)alanine	32	77	189	250	—	—	—	58
ε-*N*-Sulphanilyl-lysine	—	16	34	60	132	423	444	9
Histidine	24	94	207	215	—	—	—	37
Tryptophan	8	34	84	149	167	180	205	27
Diaminomonocarboxylic acids:								
Lysine	8	17	44	86	198	209	215	14
Ornithine	29	73	169	175	—	—	—	51
Unclassified α-amino-acids:								
Serine	5	13	28	50	84	115	136	8
Threonine	1	3	4	6	12	20	24	2
Proline	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Piperidine-α-carboxylic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Methionine	14	52	128	221	254	—	—	40
Cystine	18	72	178	304	484	558	610	56

The amino-acid oxidase produced by this organism was found to be specific for L-amino acids. The enzyme was present in the clear medium as well as in the mycelium. Both the mycelium extract and the culture fluid contained a powerful catalase so that H_2O_2 did not accumulate.

Results

Rate of oxidation of various amino-acids

Variations were found in the potency of the enzyme prepared from different batches, and there was a slight fall in the potency of the crude extract on storage even at 0°. All the amino-acids were therefore tested on the same day with the same enzyme preparation. The enzyme solution used contained 0·57% dry matter (excluding buffer) and gave a blank oxygen absorption of 4 μl./ml. in 5 min. and 8 μl./ml. in 10 min.

The data obtained are shown in Table 3. The figures in the last column indicate the relative maximum rates of oxidation; they were calculated from the difference between either the 5 and 10 min. readings or the 10 and 20 min. readings whichever gave the higher value. This procedure was prompted by the existence of a lag period which delayed the development of the maximum rates.

Straight-chain α-aminomonocarboxylic acids. The data show that in the homologous series the C_4 and C_5 acids were oxidized more rapidly, and the C_8 acid more slowly than alanine; the higher homologues, from C_{11} upwards, were not attacked.

Branched-chain α-aminomonocarboxylic acids. Valine was oxidized very much more slowly than its straight-chain isomer. Leucine was oxidized more readily and isoleucine more slowly, than the straight-chain isomer.

Aliphatic α-aminodicarboxylic acids. Aminoadipic and aminopimelic acids were much more rapidly oxidized than aspartic and glutamic acids and the C_{13} member of this series.

Amino-acids with cyclic substituents. Tyrosine showed a lower rate of oxidation than phenylalanine. All of the amino-acids in this group were oxidized including ε-*N*-sulphanilyllysine. β-Quinolyl(4)-alanine was attacked at approximately the same rate as β-quinolyl(2)alanine.

Diaminomonocarboxylic acids. Both amino-acids tested were attacked rather slowly, ornithine reacting more rapidly than lysine.

Unclassified amino-acids. The β-hydroxy-α-amino-acids, serine and threonine, were slowly oxidized. Methionine and cystine were oxidized at a moderate rate, but proline and piperidine-α-carboxylic acid were not attacked.

Total oxygen uptake

Several of the amino-acids with a cyclic substituent consumed oxygen in excess of the theoretical figure of 252 μl. Under the conditions of the experiment recorded in Table 3 tyrosine consumed 2·7, *p*-aminophenylalanine 1·5, *p*-dimethylaminophenylalanine 2·4 and ε-*N*-sulphanilyllysine 1·8 atoms of oxygen. In none of these cases had the oxygen consumption ceased at the end of the period of observation. In another experiment the oxygen absorption of *p*-aminophenylalanine ceased after 2 atoms of oxygen had been absorbed. In the case of tyrosine the final solution was dark brown.

Cystine also absorbed more than the expected amount of oxygen (more than 2·4 atoms per molecule).

Experiments with the D-amino-acid oxidase of *Neurospora crassa*

Procedure

The strain 'Beadle 25*a*', supplied by Dr N. H. Horowitz, was used as a source of this enzyme, which was found to be specific for D-amino-acids. The growth medium and the procedure generally was essentially the same as that of Horowitz (1944). The incubation period was 7 days, when the maximum enzyme activity was reached. Of the mycelium extract, 4 ml. were used per manometer cup.

Results

Rate of oxidation of various amino-acids

The data obtained are recorded in Table 4. All figures are corrected for the oxygen consumption of the blank control which was 20 μl. for the first 20 min./4 ml. of extract. The enzyme solution contained 2·75% dry matter excluding the buffer. The figures in the last column of Table 4, indicating the relative maximum rates of oxidation, were calculated from the oxygen uptake values for the second or third 10 min. periods, whichever gave the higher value. In general, the relative activity of the D-amino-acid oxidase is much weaker than that of the L-enzyme, as shown by the comparison of the last columns of Tables 3 and 4. In many cases q_{O_2} for the same amino-acid is 10–50 times greater in the case of the L-enzyme.

Straight-chain α-aminomonocarboxylic acids. The C_4 homologue showed the highest rate of oxidation; the C_8, C_{11} and C_{12} compounds were not attacked while the C_{18} compound, after a lag period, was slowly oxidized.

Branched-chain α-aminomonocarboxylic acids. Valine and isoleucine were oxidized more slowly and leucine more rapidly than their straight-chain isomers.

Aliphatic α-aminodicarboxylic acids. Glutamic acid showed the highest rate. It is further noteworthy that the C_7 acid reacted more rapidly than the C_6 acid. In the case of aspartic acid there was a lag period of about 30 min. before the rate became appreciable.

Table 4. *Oxidation of amino-acids by the* D-*amino-acid oxidase of* Neurospora crassa (*Strain Beadle 25a*)

(For conditions and calculations of q_{O_2} see text. All data are corrected for the blank O_2 absorption of the enzyme solution.)

Amino-acid	O_2 absorbed (μl.) after 10 min.	20 min.	30 min.	60 min.	120 min.	q_{O_2}
Straight-chain α-aminomonocarboxylic acids:						
Alanine	7	21	33	67	103	0·8
α-Aminobutyric acid	40	94	139	192	—	3·0
α-Aminovaleric acid	35	75	113	233	—	2·2
α-Aminocaproic acid	4	17	29	82	179	0·8
α-Aminocaprylic acid	Nil	Nil	Nil	Nil	Nil	Nil
α-Aminohendecanoic acid	Nil	Nil	Nil	Nil	Nil	Nil
α-Aminolauric acid	Nil	Nil	Nil	Nil	Nil	Nil
α-Aminostearic acid	0	2	3	11	35	0·1
Branched-chain aliphatic α-aminomonocarboxylic acids:						
Valine	0	4	7	21	45	0·2
Leucine	27	55	81	167	205	1·5
Isoleucine	5	8	9	27	59	0·2
Aliphatic α-aminodicarboxylic acids:						
Aspartic acid	0	0	5	29	57	0·3
Glutamic acid	10	23	36	78	143	0·7
α-Aminoadipic acid	0	1	5	12	22	0·2
α-Aminopimelic acid	5	14	20	36	55	0·5
1-Aminohendecane-1:11-dicarboxylic acid	Nil	Nil	Nil	Nil	Nil	Nil
α-Amino-acids with cyclic substituents:						
Phenylalanine	26	53	76	167	238	1·2
Tyrosine	0	6	8	27	55	0·3
p-Aminophenylalanine	0	0	0	17	31	—
p-Dimethylaminophenylalanine	0	12	24	81	173	—
p-(β-Aminoethyl)phenylalanine	59	124	179	208	286	3·5
p-Guanidino-methylphenylalanine	17	40	65	133	188	1·3
β-Quinolyl(4)alanine	Nil	Nil	Nil	Nil	Nil	Nil
β-Quinolyl(2)alanine	Nil	Nil	Nil	Nil	Nil	Nil
β-6-Methoxyquinolyl(4)alanine	10	17	25	48	93	0·4
β-Pyridyl(4)alanine	43	95	147	222	298	2·8
β-Furyl(2)alanine	52	113	176	247	—	3·4
ε-*N*-Sulphanilyllysine	Nil	Nil	Nil	Nil	Nil	Nil
Histidine	0	1	10	47	87	0·5
Tryptophan	Nil	Nil	Nil	Nil	Nil	Nil
Diaminomonocarboxylic acids:						
Lysine; ornithine	Nil	Nil	Nil	Nil	Nil	Nil
Unclassified α-amino-acids:						
Serine; threonine; proline; piperidine-α-carboxylic acid and cystine	Nil	Nil	Nil	Nil	Nil	Nil
Methionine	46	101	155	246	—	3·0

Amino-acids with cyclic substituents. Several of these substances were rapidly oxidized. Considerable lag periods were noted in the case of tyrosine, *p*-aminophenylalanine, and histidine. In some cases the total oxygen uptake exceeded the calculated amount, and this was further examined in additional experiments where the oxygen uptake was measured over a period of 4 hr. An excess oxygen absorption was found with *p*-(β-aminoethyl)phenylalanine, *p*-aminophenylalanine, and *p*-dimethylaminophenylalanine, pyridyl(4)alanine and tyrosine.

Diaminomonocarboxylic acids. Neither of two substances tested was attacked.

Unclassified amino-acids. Methionine was more rapidly oxidized than any of the other naturally occurring amino-acids, as already noted by Horowitz (1944). Serine, threonine, cystine and proline were not attacked.

DISCUSSION

Comparison of the specificity of the four amino-acid oxidases. Data suitable for the comparison of the activities of the four enzymes studied are those given in the last column of Tables 1, 3 and 4 and the 60 min. values in Table 2. These figures all represent the oxygen uptake per mg. dry wt. of enzyme preparation per hour. Some comparative data are listed in Table 5. The relative rates at which the different amino-acids undergo oxidation vary considerably from enzyme to enzyme. For example, of the five monoaminodicarboxylic acids tested only the C_{13} compound is attacked by the cobra-venom

Table 5. *Comparison of the reactivity of amino-acids with the four different amino-acid oxidases*

Type of amino-acid	D-Amino-acid oxidase of sheep kidney	L-Amino-acid oxidase of cobra venom	L-Amino-acid oxidase of *N. crassa*	D-Amino-acid oxidase of *N. crassa*
Straight-chain aliphatic monoaminomonocarboxylic acids	$C_8 > C_6 > C_4 > C_5 > C_8$	$C_6 > C_8 > C_5$	$C_4 > C_5 > C_6 > C_3 > C_8$	$C_4 > C_5 > C_6$, $C_8 > C_{18}$
	C_{11}, C_{12}, C_{18} not attacked	C_3, C_4, C_{11}, C_{12}, C_{18} not attacked	C_{11}, C_{12}, C_{18} not attacked	C_8, C_{11}, C_{12} not attacked
Branched-chain monoaminomonocarboxylic acids	Valine > isoleucine > leucine	Only leucine oxidized	Leucine > isoleucine > valine	Leucine > isoleucine, valine
Aliphatic monoaminodicarboxylic acids	C_{13} rapidly oxidized	Only C_{13} oxidized	$C_7 > C_6 > C_{13} > C_5 > C_4$	$C_5 > C_7 > C_4 > C_6$
	C_4 slowly oxidized	—	All oxidized	C_{13} not attacked
	C_5, C_8 and C_7 not attacked	—	—	—
Diaminomonocarboxylic acids	Ornithine > lysine	Not attacked	Ornithine > lysine	Not attacked
Amino-acids with cyclic substituent	Tyrosine > aminophenylalanine > dimethylaminophenylalanine > phenylalanine	Tyrosine > aminophenylalanine and phenylalanine > dimethylaminophenylalanine	Dimethylaminophenylalanine > phenylalanine > aminophenylalanine > tyrosine	*p*-Aminophenylalanine > β-pyridyl(4)alanine > phenylalanine > tyrosine
	Dimethylaminophenylalanine consumed > 1 atom O	Tyrosine, aminophenylalanine, dimethylaminophenylalanine, tryptophane and β-furyl(2)-alanine consumed > 1 atom O	Tyrosine, aminophenylalanine, dimethylaminophenylalanine, ε-*N*-sulphanilyllysine consumed > 1 atom O	*p*-(β-Aminoethyl)phenylalanine, *p*-aminophenylalanine, *p*-dimethylaminophenylalanine, pyridyl(4)alanine, tyrosine consumed > 1 atom of O
	ε-*N*-Sulphanilyllysine not attacked			
	β-Quinolyl(2)alanine > β-quinolyl(4)alanine	Histidine and ε-*N*-sulphanilyllysine not attacked	—	
	—	β-Quinolyl(4)alanine > β-quinolyl(2)alanine	β-Quinolyl(4)alanine and β-quinolyl(2)alanine attacked at similar rates	—
Unclassified amino-acids	Proline > methionine > serine > threonine > cystine	Only methionine oxidized	Serine > cystine > methionine > threonine	Only methionine attacked
	—	—	Proline and piperidine-α-carboxylic acid not attacked	—
	—	—	Cystine consumed > 1 atom O	—
Total number of amino-acids oxidized out of 38 tested	31	19	33	23

oxidase, whilst the D-amino-acid oxidase of kidney attacks the C_4 and C_{13} compounds but not the C_5, C_6 and C_7 homologues. The L-enzyme of *Neurospora* oxidizes all five substances with the maximum rate for the C_5 compound; the D-enzyme of *Neurospora* oxidizes the C_4, C_5, C_6 and C_7 but not the C_{13} compounds with the maximum rate for the C_5 compound.

Proline and piperidine-α-carboxylic acid are attacked by the kidney D-amino-acid oxidase only, and ε-*N*-sulphanilyllysine by the L-enzyme of *Neurospora* only. Another example of the different behaviour of the four enzymes is given by the relative reactivity of β-quinolyl(4)alanine and β-quinolyl(2)alanine. The kidney D-amino-acid oxidase oxidizes the latter much more rapidly than the former. The L-enzyme of *Neurospora* oxidizes both compounds rapidly at about the same rate, but the D-enzyme of *Neurospora* does not attack these substances. Similar differences could be enumerated for other series of amino-acids. It might be especially emphasized that the D- and L-enzymes of *Neurospora* differ not only in their optical specificity but also in many other properties. Common to all four enzymes is the inability to attack α-amino-acids with a tertiary α-carbon and β-substituted aspartic acids.

The findings lead to the general conclusion that among the factors which determine the oxidizability of α-amino-acids the specific properties of the enzyme are no less important than the structure of the amino-acid molecules.

Lag periods. The comparison of the reaction rates is in many cases complicated by the occurrence of a lag period. With kidney D-amino oxidases this was usually of the order of a few minutes; with the two *Neurospora* enzymes it varied between a few minutes and about 1 hr. Lag periods of this kind are generally ascribed to the formation, by the initial process, of a new reactive substance, either from the substrate or from the catalyst. A more specific hypothesis to explain the lag period for the enzymes under discussion is so far lacking.

Comparison with previous observations. Considering differences in experimental conditions the data reported in this paper may be regarded as agreeing essentially with those recorded in the literature for the naturally occurring amino-acid (for kidney D-amino-acid oxidase see Krebs, 1935, 1948; Felix & Zorn, 1939; Felix, 1947; Kögl, Herken & Erxleben, 1940; Klein & Handler, 1941; for cobra-venom L-amino-acid oxidase see Zeller, 1948; for *Neurospora* D-amino-acid oxidase see Horowitz, 1944). A few discrepancies occur in cases where the rate of oxidation, and therefore the accuracy of the measurements, is low.

SUMMARY

1. The rate of oxidation was examined of forty-eight α-amino-acids, among them twenty-seven synthetic compounds not previously tested, in the presence of crude preparations of the D-amino-acid oxidase of sheep kidney, of the L-amino-acid oxidase of cobra venom and the L- and D-amino-acid oxidases of *Neurospora crassa*. Data are given for the rates of oxidation.

2. None of the enzymes attacked α-amino-acids with a tertiary α-carbon atom, or β-substituted aspartic acids. Of the remaining thirty-eight amino-acids tested the kidney D-amino acid oxidase attacked thirty-one compounds, the cobra-venom L-enzyme nineteen, the *Neurospora* L-enzyme thirty-three, and the *Neurospora* D-enzyme twenty-three.

3. The specificity of the four enzymes showed many differences. The D- and L-enzymes of *Neurospora* differed not only in the optical specificity but also in many other properties.

4. Lag periods during which the rate of reaction increased were observed in the case of the kidney D-amino-acid oxidase and of the two *Neurospora* enzymes.

5. A number of amino-acids with cyclic substituents absorbed more oxygen than required for the conversion into the keto acid. This applied to *p*-dimethylaminophenylalanine with all four enzyme preparations, to tyrosine and *p*-aminophenylalanine with the cobra enzyme and the two *Neurospora* enzymes, to tryptophan and β-furyl(2)alanine with the cobra enzyme, ε-*N*-sulphanilyllysine with the *Neurospora* L-enzyme, to *p*-(β-aminoethyl)-phenylalanine and β-pyridyl(4)alanine with the *Neurospora* D-enzyme.

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The Biosynthesis of Prodigiosin, the Tripyrrylmethene Pigment from *Bacillus prodigiosus* (*Serratia marcescens*)

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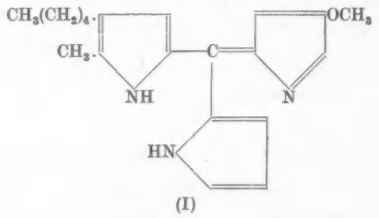
Following the pioneer work of Schoenheimer, relatively simple organic compounds have, in many instances, been identified as precursors of much larger and more complicated molecules of biological importance. Efforts to bridge the gap between these small organic building stones and the complex final product have, however, met with less success. By investigating the biosynthesis of prodigiosin, the only known naturally occurring tripyrrylmethene, we hoped not only to elucidate this process for its own sake, but also to study it as a possible intermediate or side reaction in the biosynthesis of porphyrins (Rimington, 1949). A preliminary communication on this subject has already appeared (Hubbard & Rimington, 1949).

In an effort to explain the occurrence in nature of porphyrins belonging to both the I and III series of isomers, Turner (1940) postulated a tripyrrylmethene as a possible intermediate in porphyrin biosynthesis. His suggestion derived support from the earlier work of Corwin & Andrews (1936), who identified a tripyrrylmethane intermediate in the pyrrol aldehyde synthesis of dipyrrylmethenes and offered an explanation, thereby, of the unexpected occurrence of more than one dipyrrylmethene in the products of the reaction.

Prodigiosin



(I)

Since a tripyrrylmethene structure (I) was assigned to prodigiosin by Wrede & Rothhaas (1934), it seemed appropriate to study its biosynthesis and to compare the results with those obtained for haemin biosynthesis by other workers (for summary see Shemin, 1948; Muir & Neuberger, 1948).

EXPERIMENTAL

Extraction of prodigiosin

Prodigiosin was prepared from cultures of *Bacillus prodigiosus* (*Serratia marcescens*), National Collection of Type Cultures no. 4612. The cultures were grown on a medium containing Difco-proteose (1%), Lab-Lemco (1%), agar (2%), and NaCl (0·5%) in tap water. The medium (usually 1 l. distributed in ten Roux bottles) was inoculated, after sterilization, by spreading on it a thin film of a suspension of the organism in a meat-extract broth. To prepare the suspension, a red colony was picked off a stock agar slope and cultured in meat-extract broth overnight at room temperature. The inoculated Roux bottles were kept at room temperature in the dark. Pigmentation was visible after 24–48 hr., but cultures were not harvested until 4–7 days after inoculation. The pigment was extracted by one of the two following methods.

Alkaline extraction. For this procedure, Wrede & Hettche's (1929) method was slightly modified. The cells were washed off the medium with 0·9% (w/v) NaCl and solid NaOH added with constant agitation up to a concentration of 10% (w/v). After 2 hr., absolute ethanol and light petroleum (b.p. 40–60°) were added in amounts roughly equal to the volume of the solution. The mixture was shaken thoroughly, and the lower layer drawn off. Both layers were then repeatedly re-extracted with light petroleum until no more pigment could be removed. The light petroleum extracts were pooled for further purification. The cell residue was so degraded by the alkali that it had to be discarded. This method, was therefore used only when no isotope analysis was planned.

Acid extraction. The culture was washed off the agar with acetone containing 10% (v/v) of 3N-HCl (in later experiments 2% (v/v) of glacial acetic acid) and was repeatedly extracted by mechanical shaking for 30–45 min. followed by centrifugation. The extracts were pooled and the cell residue was washed three times with acetone to remove all traces of acid and once with ether and then dried in a vacuum desiccator. For isotope analysis, the cells were at first extracted continuously with acetone (Soxhlet) to remove all traces of pigment and fat. After it was found that extracted and unextracted cells gave identical results, such further extraction was omitted. The total acidic acetone extract was diluted with an equal volume of distilled water and made alkaline by addition of 10% (w/v) NaOH until the colour changed from deep red to yellow-orange; it was then extracted with several portions of light petroleum. When no more pigment could be extracted, the aqueous acetone layer was discarded and the light petroleum extracts pooled.

Purification of prodigiosin

The method of purification adopted depended on whether the material was to be used for ^{15}N analysis, for isotopic C analysis or for crystallization. Crystallization was found to be far too wasteful of material to be practicable for runs containing isotopes. Satisfactory and fairly quantitative purification procedures were, however, elaborated and applied as follows:

Procedure for ^{15}N analysis. The pigment in light petroleum was extracted with 85% (v/v) ethanol containing 1% glacial acetic acid until no more could be removed. The deep-red ethanolic solution was then diluted with an equal volume of water, treated with aqueous NaOH until the colour changed to orange, and extracted repeatedly with $CHCl_3$. The combined $CHCl_3$ extracts were concentrated to a small volume under reduced pressure and slight warming, and the optical density at the maximum (540 mμ.) as well as the N (micro-Kjeldahl) content of the sample were determined. The optical density was measured with the Beckman spectrophotometer. In later experiments, N determinations were omitted.

For isotope analysis, the purified samples of pigment were evaporated to dryness in Kjeldahl digestion flasks and digested overnight with 50% (v/v) H_2SO_4 containing 1% (w/v) SeO_2 with addition of catalytic amounts of $CuSO_4$ and K_2SO_4. The samples were then distilled and analysed for ^{15}N. Analyses for ^{15}N were always performed on duplicate samples.

Procedure for ^{13}C or ^{14}C analysis. It was found that although the above purification procedure removed all nitrogenous contaminants, enough fatty material remained to make C analyses unreliable. The pigment was therefore further purified by warming under reflux for 1 hr. at about 60° in 85% (v/v) ethanol containing 10% (w/v) NaOH. The alkaline ethanolic solution was then diluted with an equal volume of water and extracted with purified light petroleum. The transfer between slightly acidic 85% ethanol and light petroleum, after dilution and addition of alkali, was repeated until the light petroleum, after extraction of the pigment, showed no trace of a fatty residue on evaporation. The pigment was then transferred once more to light petroleum, the solution concentrated to 1–2 ml. and evaporated to dryness in a small weighing vessel. The vessel with the pigment was weighed and the pigment transferred to a small volumetric flask with a few drops of glacial acetic acid. The vessel was then dried in a vacuum desiccator containing pellets of KOH and reweighed. The pigment was diluted to a given volume with $CHCl_3$ and the optical density at the maximum (540 mμ.) measured. The pigment was considered sufficiently pure if the extinction coefficient on a weight basis fell within 15% of the value calculated on the basis of N content. It was then transferred to a small tube, dried overnight in a vacuum desiccator and analysed for isotopic C. The yield was approximately 10 mg. from 1 l. of culture medium (ten Roux bottles).

Procedure for crystallization. For easy crystallization, it was necessary to remove most of the fatty contaminants. This was accomplished either by the alkaline extraction procedure (p. 220) or by the acid extraction followed by the same purification steps as for carbon analysis. The pure dry sample of pigment was dissolved in about 1 ml. or less of hot absolute ethanol and was treated while hot with a few drops of 5% (v/v) aqueous $HClO_4$ to the point of slight turbidity. The solution was then allowed to cool to room temperature and stored overnight at −10°. The purple crystals of the perchlorate came out readily and another crop could be obtained from the mother liquor after further concentration and addition of some drops of 1% (v/v) aqueous $HClO_4$. For recrystallization, the crystals were dissolved in about 0·5 ml. of hot absolute ethanol and the hot solution treated with a few drops of 1% (v/v) aqueous $HClO_4$.

Miscellaneous procedures

Estimation of glycine. Glycine in the medium was determined by the method of Alexander, Landwehr & Seligman (1945).

Preparations of labelled compounds. DL-Leucine labelled with ^{15}N was prepared by the method of Schoenheimer & Ratner (1939). Glycine, labelled with ^{13}C on the carboxyl and with ^{14}C on the methylene group respectively were kindly made available by Drs Neuberger, Bentley and Arnstein, of the National Institute for Medical Research, Hampstead, N.W. The isotopic acetates were supplied by the National Chemical Laboratory, Teddington and the NH_4NO_3 by the Tracer elements Subcommittee of the Medical Research Council. The chloride was prepared from the nitrate. Analar reagents were used throughout the experiments and light petroleum was specially purified by repeated washing with conc. H_2SO_4 followed by washing with distilled water and redistillation. All taps in contact with the prodigiosin solutions were greased with a silicone grease (Dow-Corning Co., U.S.A.)

RESULTS

Spectral properties of prodigiosin

Since there are no detailed data available on the spectral absorption of prodigiosin, and since the only previous attempt to determine its extinction coefficient quantitatively (Ehrismann & Noethling, 1936) failed for technical reasons, we shall report our investigations on the striking spectral behaviour of prodigiosin in some detail.

Prodigiosin in solution can exist in one of two distinct but readily interconvertible forms or as a mixture of these two forms, depending on the hydrogen ion concentration of the medium. The two extreme spectra are illustrated in Fig. 1 which shows the difference in the position of the absorption maxima as well as in the width and intensity of the absorption bands of the two forms. In acid solution, the pigment is red, due to a sharp, high, narrow main band with a maximum at 535–540 mμ. and a slight shoulder on the low wavelength limb of the curve at about 510 mμ. This hump is always present and is independent of the purity of the sample. In alkaline solution, the pigment is orange-yellow due to a broader, less intense, roughly symmetrical band centered at about 470 mμ. The secondary hump at 530 mμ. (Fig. 1) is due to a trace of the 535 mμ. chromogen. Transition forms between these two extremes have been studied and there appears to be a definite isobestic point at about 495 mμ. showing that the two forms are probably the only participants

in this reversible reaction. As the ordinate in Fig. 1 is the same for both curves, it can be seen that the optical densities at the maxima of the two chromogens are in the ratio of about 2·5 : 1.

Since the pigment is soluble only in non-aqueous media, the pK associated with this colour change has not been accurately determined. It is, however, certainly on the alkaline side of neutrality, probably between pH 8·5 and 9·5. In partitions between aqueous ethanol and light petroleum, the behaviour of prodigiosin depends on the acidity of the ethanol phase. With acid aqueous ethanol and light petroleum, the 540 mμ. chromogen is hypophasic down to a concentration of 30% ethanol, beyond which point the aqueous solution is too turbid for estimations of optical density. The 470 mμ. chromogen, on the other hand, is always epiphasic to alkaline ethanol. This distinction forms the basis of the method of purification by repeated partitions described above (p. 221).

The pure 470 mμ. band can only be obtained in fairly alkaline solvents, pyridine giving a mixture of the two bands. The pure 540 mμ. band is easily obtained in all non-alkaline organic solvents and

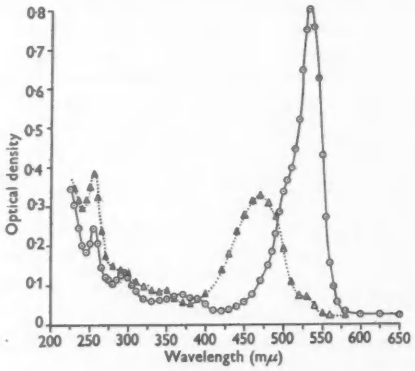


Fig. 1. Spectral properties of prodigiosin: ⊙, in 85% ethanol; △, in 85% ethanol + NH_4OH.

seems to be the more stable form as the 470 mμ. chromogen passes into the 540 mμ. chromogen spontaneously on prolonged standing. This transition is not appreciably accelerated by bubbling a stream of carbon dioxide through the solution but can be accomplished instantaneously by adding a drop of acid and somewhat more slowly by vigorous mechanical agitation, but not by bubbling oxygen through the liquid. A 540 mμ. band is also characteristic of the zinc complex of prodigiosin as well as of prodigiosin perchlorate.

The extinction coefficient of the 540 mμ. chromogen has been measured in chloroform containing 1% glacial acetic acid on the basis of nitrogen content as well as dry weight. As the figures on the basis of nitrogen have been consistently about 15% higher, they have been considered more reliable and they yield a value for ϵ (mol. extinction coefficient) of 110,000 ± 5000. The ϵ value of the 470 mμ. chromogen is therefore about 44,000.

It is difficult to interpret these spectrophotometric data chemically, since almost nothing is known concerning the spectral behaviour of tripyrrylmethenes. Dipyrrylmethenes are known to exist in two spectral forms with absorption properties similar to those described above (Granick & Gilder, 1947). However, it is safe to assume from the pK value quoted above that the reversible spectral shift is due to the formation of a sodium salt on one of the pyrrole nitrogens.

Comparison of the spectra of prodigiosin and of another tripyrrylmethene

As the structural formula of Wrede & Rothhaas (1934) for prodigiosin has not been confirmed by synthesis, it seemed worth while to study the spectral properties of one of the two tripyrrylmethenes

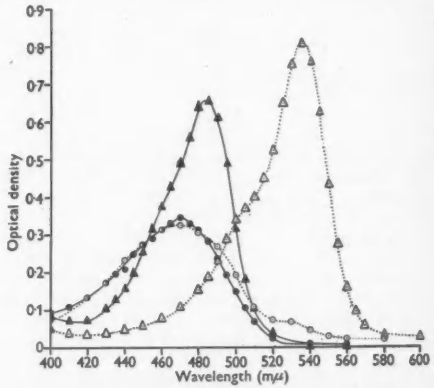


Fig. 2. Spectral comparison of prodigiosin and Fischer & Gangl's (1940) tripyrrylmethene; ▲—▲, tripyrrylmethene in acid ethanol; ●—●, tripyrrylmethene in alkaline ethanol; △ . . . . △, prodigiosin in acid ethanol; ⊙ . . . . ⊙, prodigiosin in alkaline ethanol.

synthesized by Fischer & Gangl (1940). We were fortunate to obtain a small sample of the perchlorate of Fischer & Gangl's 4-bromo-2:3′:3″-tricarbethoxy-3:2′:4′:2″:4″-pentamethyltripyrrylmethene, through the generosity of Dr Siedel, and were able to measure its spectrum in acid and alkaline ethanolic solution and compare this with the spectrum of prodigiosin. Fig. 2 presents the comparison, using the same ordinate and abscissa for all four curves. The striking similarity, if not complete identity, of the

two alkaline spectra is immediately apparent. There is also a qualitative similarity in the change of the spectra on addition of acid. In both cases the band becomes narrower and more intense and acquires a slight hump on the short wavelength limb of the absorption curve. However, whereas in the case of prodigiosin, this change is accompanied by a shift of about 65 mμ. toward longer wavelengths, there occurs only a slight shift of about 15 mμ. with the synthetic tripyrrylmethene. This difference is very likely attributable to the different ring substituents in the two compounds. Although the interpretation of these similarities should not be pushed too far, they lend definite support to the tripyrrylmethene formula proposed for prodigiosin.

The biosynthesis of prodigiosin

Nitrogen precursors. Since it has been shown (Shemin, 1948; Muir & Neuberger, 1948) that glycine is a specific precursor for all four pyrrole nitrogens of the porphyrin ring, we investigated its utilization for the biosynthesis of prodigiosin, and compared the utilization found with that of a few other nitrogenous substances. Table 1 summarizes the results. It will be seen clearly that glycine is a specific precursor for the pyrrole nitrogen of prodigiosin, whereas all the other compounds tested are utilized for prodigiosin synthesis to the same extent or less than for the synthesis of cellular protein. The efficiency of glycine utilization is 74%, i.e. of the 6% excess ^{15}N fed, about 4·5% excess appears as prodigiosin ^{15}N. This highly efficient utilization makes it seem likely that glycine is the only specific nitrogen precursor for all three pyrrole rings of prodigiosin.

Carbon precursors. In this series of experiments, the utilization of the two carbon atoms of glycine and of acetate was tested. The results are summarized in Table 2. They show that both carbon atoms of acetate are utilized at a higher level for prodigiosin synthesis than for the synthesis of the non-fatty cellular constituents. The two carbon atoms seem to be roughly equipotent as prodigiosin precursors. With glycine as a source of carbon atoms, however, only the α-carbon is utilized and this with a high degree of efficiency; the carboxyl carbon is not used at all. It is not possible to compare the acetate and glycine carbon results quantitatively since our baseline of utilization for non-fatty cellular constituents is not comparable for glycine, a protein precursor, and acetate, a fat precursor. It should, however, be stressed that acetate is definitely specifically used here as in the case of porphyrin biosynthesis.

The utilization of glycine. Table 3 gives in summary form a comparison of the utilization of the nitrogen and α-carbon atoms of glycine for prodigiosin biosynthesis. As mentioned above, the carboxyl carbon atom of glycine is not used. The results in Table 3 show that the nitrogen and α-carbon atoms are used to the same extent and with the same efficiency for prodigiosin biosynthesis and that, therefore, the nitrogen and α-carbon atoms seem to be incorporated together. Decarboxylation must occur at some stage in the formation either of the pyrrole ring or of

Table 1. *Utilization of* ^{15}N *by* Bacillus prodigiosus

Isotope supplied (Constant amount of N added)		Isotope (^{15}N) utilized		
Compound	^{15}N content (atom % excess)	Cell protein (atom % excess)	Prodigiosin (atom % excess)	Prodigiosin-^{15}N / Cell protein-^{15}N
Glycine	32	1·64	4·43*	2·70
Glycine	32	1·83	4·74*	2·59
NH_4NO_3	31·5	1·46	0·868	0·59
NH_4Cl	31·5	1·63	1·60	0·98
DL-Leucine	31·2	1·08	0·442	0·41

* Glycine content of medium reduces ^{15}N supplied as glycine to 6·0 atom percentage excess. Therefore the efficiency of incorporation of glycine $^{15}N = 74\%$.

Table 2. *Utilization of isotopic carbon* (*C) *by* Bacillus prodigiosus

Isotope supplied			Isotope utilized		
Compound	Amount added (g.)	Isotope content (atom % excess or counts/min./mg. of C)	Cell protein *C (atom % excess or counts/min./mg. of C)	Prodigiosin *C (atom % excess or counts/min./mg. of C)	Prodigiosin *C / Cell protein *C
$CH_3.{}^{13}COONa$	2·0	8	0·52	1·26	2·52
$CH_3.{}^{13}COONa$	0·5	8	0·22	0·67	3·04
$^{13}CH_3.COONa$	1·25	8	0·69	1·25	1·83
$NH_2.CH_2.{}^{13}COOH$	0·40	27	0·27	0	0
$NH_2.{}^{14}CH_2.COOH$	0·39	17,000 (counts/min./mg. of C)	865 (counts/min./mg. of C)	2080 (counts/min./mg. of C)	2·40

Table 3. *Utilization of* $NH_2{}^{14}CH_2.COOH$ *and of* $^{15}NH_2.CH_2.COOH$ *by* Bacillus prodigiosus

(Basal medium contains 1·6 g. of normal glycine.)

Isotope supplied				Isotope utilized					
^{14}C-Glycine		^{15}N-Glycine		Cell protein		Prodigiosin			
Added (g.)	^{14}C content (counts/min./mg. of C)	Added (g.)	^{15}N content (atom % excess)	^{14}C content (counts/min./mg. of C)	^{15}N content (atom % excess)	^{14}C content (counts/min./mg. of C)	^{15}N content (atom % excess)	^{14}C Prodigiosin / Cell protein	^{15}N Prodigiosin / Cell protein
0·39	17,000	0·04	32	865 (efficiency 26·4 %)	0·189 (efficiency 30·0 %)	2080 (efficiency 63·6 %)	0·444 (efficiency 70·4 %)	2·40	2·35

the tripyrrylmethene structure, but it is noteworthy that deamination reactions leading to the incorporation of residues derivable from glycine do not occur in the formation of prodigiosin, although they do so in the biosynthesis of haemin (Radin, Rittenberg & Shemin, 1949; Muir & Neuberger, 1949).

DISCUSSION

In recent years, studies of the biosynthesis of large molecules have revealed that glycine and acetate are among the most versatile small molecules which take part in synthetic processes (for review see Bentley, 1949). The discovery of yet another large molecule which is built from these two precursors therefore raises the question whether one can assume some parallelism in the mechanism of these various biosynthetic processes; in other words, whether there exist certain fundamental and general processes of chemical biosynthesis in which these simple molecules play a prominent role, and which may be adapted and elaborated to provide molecules which when completed bear little formal structural similarity to one another. As an example of such a line of thought, already familiar in biochemistry, may be mentioned the potentialities attributed to isoprene units in the synthesis, by polymerization, etc., of the mono-, di- and tri-terpenes and even other types of equally complex isocyclic structures. It is, however, also possible that the discovery of so many biosynthetic processes involving glycine and acetate may be merely fortuitous. Be this as it may, the realization that prodigiosin and the porphyrins are synthesized from the same precursors inevitably raises the question whether in porphyrin synthesis there is first formed a common pyrrole precursor which by further reaction gives rise to the cyclic structure or whether pyrrole and porphyrin rings are formed in a single event (Rimington, 1949). It is noteworthy in this connexion that there are some quantitative differences between porphyrin and prodigiosin synthesis which, however, can probably be explained by the differences in the structure of the two molecules. Thus Radin *et al.* (1949) have shown that in porphyrin biosynthesis six methyl carbons of acetate are used for every carboxyl carbon, whereas in our experiments, the two carbon atoms of acetate seem to be used to about the same extent. This difference may find its explanation in the fact that acetate is almost certainly utilized in the formation of the ring substituents which are quite different and considerably more complex in the case of the porphyrins than in prodigiosin. Similarly, Muir & Neuberger (1949) have shown that the α-carbon atom of glycine is utilized to more than twice the extent that the nitrogen atom is in porphyrin biosynthesis, whereas we have shown above that there is a strict 1:1 relationship between the utilization of the nitrogen and α-carbon atoms of glycine. This difference is almost certainly due to the fact that, as Muir & Neuberger have postulated, there is a rough 1:1 relation in the nitrogen and α-carbon content of the pyrrol rings, but they, and also London, Shemin, West and Rittenberg (1949), have shown that all the methyne bridges of the porphyrin molecule are derived from the α-carbon atom of glycine. Since the over-all architecture of the prodigiosin molecule is quite different from that of the porphyrins, this may account for the discrepancy in the carbon : nitrogen ratios.

On the basis of evidence presented above, it does not seem possible to decide definitely whether there exists a common precursor for all porphyrins as well as prodigiosin or whether each is synthesized individually *ab initio*, but the data strongly suggest some interrelation between the biosyntheses of the various pyrrole compounds. Although our experiments offer no proof of the formation of a tripyrryl intermediate, they do not exclude this possibility and we feel that the striking similarities between prodigiosin and porphyrin biosynthesis must be given due consideration in the elaboration of any final scheme of porphyrin biogenesis.

SUMMARY

1. Methods have been evolved for the isolation of prodigiosin and of bacterial cell residues suitable for isotopic analysis.

2. The absorption spectrum of prodigiosin in various solvents has been studied quantitatively, and the existence of two spectral forms depending upon the pH of the solvent demonstrated. In acid chloroform, prodigiosin has a band at 535–540 mμ. with $\epsilon = 110{,}000 \pm 5000$.

3. A comparison has been made of the spectral properties of prodigiosin and of a tripyrrylmethene synthesized by Fischer & Gangl (1940). This comparison supports the tripyrrylmethene structure assigned to prodigiosin by Wrede & Rothhaas (1934).

4. Both the nitrogen and α-carbon atoms of glycine are utilized specifically and to the same extent with a high degree of efficiency in the biosynthesis of prodigiosin. The carboxyl carbon atom of glycine is not utilized. All three nitrogen atoms of prodigiosin must be derived from glycine.

5. Both carbon atoms of acetate are utilized specifically and to about the same extent in the biosynthesis of prodigiosin.

6. The nitrogen of DL-leucine and of ammonium salts is incorporated into prodigiosin to the same extent as into the bacterial protein.

7. The experiments recorded show a striking similarity between the modes of synthesis of prodigiosin and porphyrin pigments but offer no conclusive evidence relative to the participation of a tripyrrylmethene intermediate in the formation of the porphyrin ring.

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Oral Toxicity and Lipotropic Potency of the Triethyl Homologue of Choline

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Interest in the triethyl homologue of choline arose in 1936 before tracer elements were available as aids in the study of the intermediary metabolism of compounds of physiological interest. Channon & Smith (1936) desired to test the hypothesis that dietary choline exerts its lipotropic effect by favouring the synthesis of lecithin, which in turn might speed up the transport of fat from the liver. They sought a compound resembling choline which would possess lipotropic properties and which might, if incorporated into the molecule of liver lecithin, be identified. Several homologues of choline were prepared and tested for ability to prevent the excessive accumulation of fat always seen in the livers of rats on hypolipotropic diets (Channon, Platt & Smith, 1937). Most of these compounds exhibited considerable toxicity when given by mouth, but one of them, triethyl-2-hydroxyethylammonium chloride (hereafter referred to for brevity as TECl) could be given to rats in sufficient amount to produce the hoped for decrease in liver fat. Males did not always respond as well as females and were, in some cases, relatively unresponsive.

Although TECl had been shown to possess definite lipotropic activity, Channon, Platt, Loach & Smith (1937) were unable to detect the presence of this homologue of choline in the liver phospholipids of rats given about 11·5 mg. daily for 20 days. Higher dosage was avoided because of the toxicity of the compound. Ten years later the validity of their attractive hypothesis received support when McArthur (1946) succeeded in establishing the presence of the triethyl homologue of choline in the liver phospholipids of rats fed TECl. Meanwhile, the lipotropic activity of TECl had been confirmed and the absence of lability of the terminal ethyl groups was established, as expected, when TECl was found to be incapable of supporting the growth of young rats on diets containing homocystine as the only source of amino-acid sulphur (du Vigneaud, Chandler, Moyer & Keppel, 1939; Moyer & du Vigneaud, 1942). TECl was found to prevent the development of haemorrhagic kidneys in young rats (Welch, 1941), but it does not protect poultry against perosis or permit their growth (Jukes, 1941).

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McArthur, Lucas & Best (1947) described in detail the conditions under which the incorporation of TECl into liver phospholipids was established. Their rats were given about six times as much of the choline homologue per day for twice as long a period without occasioning as much loss of body weight as was found by Channon, Platt, Loach & Smith (1937).

Studies of the acute and chronic oral toxicity of TECl (made before the experiments of McArthur *et al.* were undertaken), lipotropic dose-response curves of TECl and of choline chloride, hitherto unpublished, and protocols of an experiment in which weanling rats were given TECl in place of choline chloride are presented briefly in the following pages.

EXPERIMENTAL

TECl used in the present studies was made in a yield of 60–75% from carefully redistilled ethylene chlorohydrin (British Drug Houses, fraction boiling at 127–128°) and triethylamine (Eastman Kodak, fraction boiling at 88–89°) as described in detail elsewhere (McArthur *et al.* 1947). After recrystallization from absolute ethanol (0·5 ml./g.), acetone (1 ml./g.) and anhydrous ether (5 ml./g.) the product was tested for contamination with triethylamine hydrochloride by boiling a portion in the Parnas-Wagner micro-Kjeldahl apparatus with strong NaOH. If any volatile base distilled over in 3 min., the product was recrystallized.

Acute oral toxicity was determined by giving the TECl (generally in 1·00 ml. of solution) by stomach tube. Rats of both sexes, weighing between 150 and 160 g. were used. In a preliminary test, groups of three rats were given 25, 50, 100 and 200 mg., respectively. One death only, and that in the final group, was obtained. Groups of three rats were then given 400, 600 and 800 mg. of TECl, respectively, as shown in Table 1 and Fig. 1, where all the data from this and subsequent tests are summarized. The effect of concentration, as distinct from absolute amount of drug, was examined by giving some of the test doses in 2·00 ml. Choline chloride was given to other rats under identical conditions. No special diet was fed to the rats used for these acute oral toxicity studies. Food and water were withheld for 1 hr. previous to the test dose and for about 3 hr. following administration of the drug.

The chronic toxicity studies were conducted with groups of five rats (150–170 g.) fed on a diet of the following percentage composition: casein, 8; gelatin, 12; salts (McCollum No. 185), 5; agar, 2; sucrose, 41; 'vitamin powder A', 2; cod liver oil concentrate (Ayerst, McKenna and Harrison), 0·010; beef dripping, 25; corn oil (Mazola), 5. 'Vitamin powder A' was a mixture in powdered sucrose of such a com-

position that at a food intake of 8 g./day each rat would receive aneurin hydrochloride (30 μg.), riboflavin (25 μg.), pyridoxin hydrochloride (20 μg.), Ca pantothenate (100 μg.) and nicotinic acid (100 μg.). Three such diets, containing 0·22, 0·45 and 0·89% TECl, respectively, were fed to the rats for 21 days, and subsequently, because of the negligible toxic effects observed, a diet containing 1·13% was fed to 20 rats for 40 days.

A similar basal diet containing 30% beef fat was used to determine the lipotropic dose-response relationship in rats weighing 70–90 g. Two separate experiments were performed with dietary fat content at 30% and one with it at 12% (beef fat 10%, corn oil 2%). A more severely hypolipotropic, but in other respects a more adequate, diet (T45) was used for a similar dose-response study with weanling rats (36–42 g.). Diet T45 has the following percentage composition: arachin, 12; gelatin, 6; casein, 3; fibrin, 1; cystine, 0·5; salt mixture (Beveridge & Lucas, 1945), 5; celluflour, 2; sucrose, 57·5; beef dripping, 10; corn oil 2; cod liver oil concentrate, 0·015; 'vitamin powder B', 1. This mixture (powder B) contained four more vitamins than did powder A. One kg. of vitamin powder B contained aneurin hydrochloride, 0·50 g.; riboflavin, 0·25 g.; pyridoxin hydrochloride, 0·20 g.; Ca pantothenate, 1·00 g.; nicotinic acid, 1·00 g.; folic acid, 0·05 g.; 2-methyl-1:4-naphthoquinone, 0·10 g.; inositol, 50 g.; *p*-aminobenzoic acid, 10·0 g. and powdered sucrose, 936·9 g.

The supplements added to these basal rations and the results are shown in Tables 2–4 and Fig. 1. It should be noted that the supplements of TECl and choline Cl were added in amounts molecularly equivalent to 2, 4, 8, 16, 32 or 64 mg. of choline (free base) per 10 g. of diet.

The rats, housed in all-metal individual cages with a false bottom of coarse wire screen, were supplied with fresh water *ad lib.* and were offered 8·0 g. of food daily; unconsumed diet was weighed each morning. The daily food consumption was recorded and the TECl or choline Cl intake per rat calculated. At the end of the experimental period the animals were stunned and the livers and kidneys were removed and weighed. When portions of these organs were taken for histological examination the remainders were reweighed and disintegrated immediately under acetone in a pyrex test tube (100 ml.) by means of a rapidly revolving micromincer, somewhat similar to one described by Alford & Palmes (1944). Best, Lucas, Patterson & Ridout (1946) have already described the extraction of total lipids with boiling ethanol, their rectification with light petroleum, and the analytical procedure used in this laboratory.

RESULTS

The data in Table 1 and Fig. 1 show that the acute toxicity of TECl administered orally to rats is slightly greater than twice that of choline chloride given under similar conditions. The effects of TECl or of choline chloride solutions administered by stomach tube were apparent, in most cases, within 15 min. Both compounds in adequate doses produced similar states of hyperexcitability, often including convulsions, followed by collapse, respiratory difficulty and eventually death within 30 min. The animals that survived frequently failed to exhibit the excitement; they appeared drowsy and remained quiescent unless disturbed. Only in a few cases did animals that survived the initial 30 min. period die within the following 16 hr. We observed, as has already been noted by Neuman & Hodge (1945), a marked difference in susceptibility of groups of rats tested at different times. For example, in February 1946, three out of three rats died on oral administration of 150 mg. of TECl in 1 ml.; in March 1948 none out of six rats died from a similar dosage.

In Table 1 are summarized the results of toxicity studies made with small groups of rats at five different times, over a period of more than 3 years. Any changes in tolerance of the population of the rat colony were probably evened out by tests over such a period.

The empirical probits corresponding to the data shown in the first six columns of Table 1 when plotted (Fig. 1) were too irregular for visual estimation of the line of best fit. The method of least squares was therefore used to determine the parameters of this provisional line from which the expected probits (Y) shown in column 8 were obtained. The method of estimating the probit regression equation by a process of successive approximations, starting with the provisional probit line, is described by Finney (1947). For the computations required to fit the probit regression line by the maximum likelihood method, the instructions given by Finney (1947, Appendix 1) were followed.

The LD_{50} of TECl appears to be 316 mg. when administered in 1 ml. of solution by stomach tube to rats weighing 150–170 g. (Dr B. Mendel and Mr D. K. Myers in this Department have estimated the LD_{50} of the same sample of TECl by intravenous and intraperitoneal injection to be about one-fiftieth of the value found when the drug was given by stomach tube. Details of their work will be published elsewhere.) Choline chloride under similar conditions has an LD_{50} of about 760 mg. when given in 2 ml. of solution.

No signs of chronic toxicity of TECl were obvious at the dosages studied. The average food consumption of the rats during the first 4 days on the three diets (7·8, 7·5 and 7·0 g., respectively) did not indicate that the diets were unpalatable. Although there was a gradual decline in food consumption, a similar decrease occurred in the control group on the drug-free basal diet. Since there was no progressive decrease in food intake or change in weight which could be related to the TECl content of the diet, it was concluded that even the largest dosage fed was without definite toxic effect. When the diet containing 1·13% TECl was fed to 20 rats for 40 days, a slight loss in weight (about 10%) was observed during the first 20 days, but after that time the weight remained essentially constant. With the food consumption averaging just over 6 g. per day during the 40-day period the daily intake of the TECl was about 70 mg. The failure of these rats to gain in

Table 1. *Estimation of median lethal dose* (LD_{50}) *of* TECl *and comparison with that of choline chloride*

(Probit regression line for: TECl, $Y = 2{\cdot}93\,x - 2{\cdot}31$, $LD_{50} = 316$ mg.; choline chloride, $Y = 10{\cdot}04\,x - 23{\cdot}97$, $LD_{50} = 760$ mg.)

Dose (mg.)	Volume (ml.)	Log dose (x)	No. of rats (n)	Deaths Obs. (r)	Deaths % (p)	Probits Empirical	Probits Expected Y	Probits Working y	Probits Max. likelihood	Calc. kill (P)	Expected deaths (nP)	Discrepancy ($r - nP$)
						TECl						
100	1·0	2·00	3	0	0	—	3·92	3·29	3·55	0·075	0·23	−0·23
150	1·0	2·18	9	3	33	4·56	4·31	4·58	4·07	0·177	1·62	+1·38
200	1·0	2·30	12	3	25	4·33	4·56	4·34	4·42	0·280	3·36	−0·36
250	1·0	2·40	22	7	32	4·53	4·77	4·54	4·72	0·390	8·58	−1·58
300	1·0	2·48	24	11	46	4·90	4·94	4·90	4·92	0·470	11·28	−0·28
350	1·0	2·54	24	13	54	5·10	5·07	5·10	5·13	0·550	13·20	−0·20
400	1·0	2·60	14	10	71	5·55	5·21	5·56	5·30	0·617	8·64	+1·36
600	1·5	2·78	3	2	67	5·44	5·59	5·43	5·83	0·797	2·39	−0·39
800	2·0	2·90	3	3	100	—	5·83	6·55	6·18	0·880	2·64	+0·36
						Choline chloride						
600	2·0	2·78	10	2	20	4·16	3·96	4·18	3·94	0·145	1·45	+0·55
800	2·0	2·90	9	4	44	4·85	5·25	4·84	5·14	0·555	4·99	−0·99
950	2·0	2·98	10	9	90	6·28	6·11	6·27	5·95	0·830	8·30	+0·70

weight is attributed to the low methionine content, or some other defect, of the basal diet and not to drug toxicity since the animals on the basal diet exhibited similar weight changes. The toxicity observed by Channon & Smith (1936) and Channon, Platt, Loach & Smith (1937) was probably attributable largely to inadequacies of their basal diet.

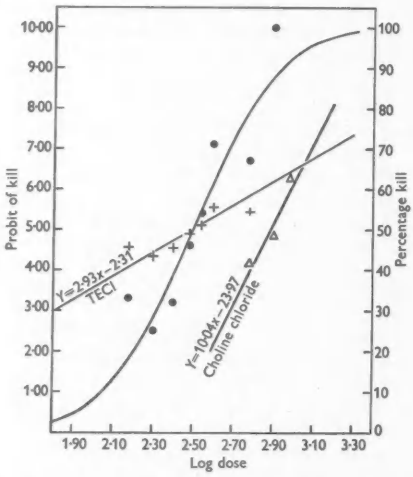

Fig. 1. Relationship between dosage of TECl and percentage kill. Observed percentage kill (●) converted to probits (+). The probit regression line was fitted by maximum likelihood method and the sigmoidal curve relating log dose to percentage kill was derived from this line. For comparison, the probit regression line for choline chloride (△) is also given.

The lipotropic dose-response curves (Fig. 2) show that the triethyl homologue of choline, although considerably less potent than choline, will, if given in sufficient amount, bring the total liver lipids down to the same normal value as does an adequate amount of choline. The data of curves *A–D* were obtained under identical dietary conditions, except that the nature and amount of fat were different in the diets fed to the rats whose liver lipids are represented by curve *A*.

Curve *A* shows the lipotropic effect of TECl in a diet containing 12% fat (beef fat 10%, corn oil 2%), and curve *B* represents the mean response from two experiments in which the diets contained 30% beef fat (Table 2). The consistently higher level of liver lipids on the lower fat intake (curve *A*) may possibly be related to change in environmental conditions rather than to the presence of highly unsaturated lipid (corn oil) along with the beef fat. Curve *C*, drawn through all experimental points available for TECl, represents the mean lipotropic effect of this compound. Curve *D* represents the lipotropic effect of choline in a diet containing 30% beef fat. Curves *B* and *D* may be compared, since they represent responses obtained simultaneously under strictly comparable conditions (30% beef fat).

Comparison of lipotropic potencies can be made only on the sloping portions of the curves, of course, since it is obvious that once such a curve tends to parallel the horizontal axis all doses beyond the shoulder of that curve will produce the same maximal response. To estimate relative lipotropic potencies the amount of each of the agents necessary to produce the same levels of liver lipids must be compared. For example, from curves *B* and *D* it may be seen that the amount of TECl molecularly equivalent to 4 mg. of choline base reduces the liver

Table 2. *Lipotropic activity of* TECl

(Test diets containing 30% beef fat fed for 21 days to groups of 10 rats, initially weighing 75–90 g. All rats group pair-fed with those on basal ration; average daily food intake in Exp. *TA* about 6·7 g.; in Exp. *TB* about 5·6 g. (except *TB*-4, in which it averaged only 4·3 g.

The supplements were molecularly equivalent to 2, 4, 8, 16, 32 or 64 mg., respectively, of choline (free base)/10 g. of food, e.g. diet *TA*-3 containing 12·0 mg. TECl/10 g. and diet *TA*-6, containing 9·2 mg. choline chloride, each contains an amount of quaternary ammonium salt corresponding molecularly to 8 mg. of choline base.)

Group no.	Supplement in terms of choline equiv. (mg./10 g.)	Average liver wt. Wet (g.)	Average liver wt. Dry, fat-free (g.)	Total liver lipids (g./liver)	Kidney Wt. (g.)	Kidney Body wt. (%)
TA-0	None (basal)	5·14	0·84	1·237	1·23	1·84
TA-1	TECl≡2	4·78	0·85	0·810	1·15	1·60
TA-2	TECl≡4	5·59	0·93	1·164	1·15	1·53
TA-3	TECl≡8	5·02	0·95	0·872	1·03	1·37
TA-4	TECl≡16	4·31	0·85	0·610	0·96	1·41
TA-5	TECl≡32	4·05	0·84	0·316	0·95	1·44
TA-6	Choline Cl≡8	4·09	0·89	0·295	1·12	1·37
TB-0	None (basal)	5·48	0·93	1·364	—	—
TB-1	TECl≡8	5·24	0·94	1·021	—	—
TB-2	TECl≡16	4·86	0·84	0·977	—	—
TB-3	TECl≡32	4·34	0·90	0·287	—	—
TB-4	TECl≡64	3·91	0·80	0·336	—	—
TB-5	Choline Cl≡4	5·20	1·02	0·609	—	—
TB-6	Choline Cl≡8	4·41	0·95	0·323	—	—
TB-7	Choline Cl≡16	4·91	1·06	0·351	—	—
TB-8	Choline Cl≡32	4·62	1·04	0·264	—	—

lipids to 21% wet weight. Choline chloride equivalent to about 0·8 mg. of choline base produced the same response. Therefore, under these conditions, choline chloride is about five times as potent as TECl on a molecular basis. Since the molecular weights of choline chloride (139·6) and TECl (181·7) are in the ratio of 1 : 1·30, the relative potencies on a weight basis are about 6·5 : 1. Similarly, the amount of TECl equivalent to 8 mg. of choline base has the same effect as choline chloride equivalent to 1·4 mg. choline base, i.e. the ratio (molecular) of potencies is about 6 : 1. However, when the amount of TECl exceeds that corresponding to about 25 mg. of choline base, both curves are approaching the horizontal and valid comparison becomes impossible, as mentioned above.

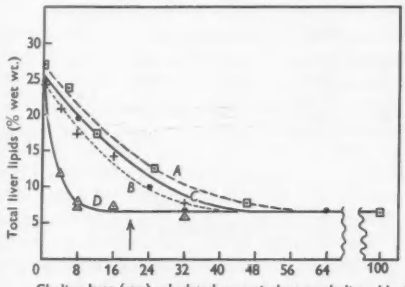

Fig. 2. Lipotropic dose-response curves. Effects of TECl (curves *A*, *B* and *C*) and of choline chloride (curve *D*) on total liver lipids when fed for 21 days. Ten rats (70–90 g.) per group. Lipid values expressed as percentage of wet tissue weight. Curve *A* (⊡), rats on diet containing 12% fat (beef fat 10%, corn oil 2%). Curve *B*, two separate experiments (+ and ●) with rats consuming diets containing 30% beef fat. Curve *C* represents the mean of all values obtained with TECl, since it is questionable whether curves *A* and *B* differ significantly. Data for curve *D* (△), showing effects of choline chloride in diet containing 30% beef fat, are comparable with those shown by +, obtained at the same time.

Channon & Smith (1936) reported that TECl is almost as potent as choline in its lipotropic effect upon female rats but that its effect is far less pronounced on bucks. The data presented here in Fig. 2 and Table 3 fail to confirm either statement. It is now obvious why Channon & Smith mistakenly concluded that choline and its triethyl homologue possess about equal activity: in the absence of a dose-response curve, the drugs were compared at dosage levels beyond that at which they exert their maximal effect, i.e. on the horizontal portion of the dose-response curve (in regions to the right of the arrow in Fig. 2). Possibly because of a difference in the strains of rats used in Liverpool and Toronto, or possibly because of differences in the nature of the basal diet, no significant difference in the response of males and females has been observed, as shown by the data in Table 3.

The effects of three different dietary levels of TECl upon weanling rats are shown in Table 4. Rats

Table 3. *Lipotropic response of male and female rats to* TECl

(Ten male and ten female rats (70–90 g.) fed with a diet containing 0·25% TECl for 21 days. Average food consumption of both groups was 6·2 g./day. Values arranged in increasing order.)

	Total liver lipids (% wet wt.)	
	Females	Males
	6·9	5·5
	7·2	9·2
	7·6	10·6
	8·0	12·5
	10·4	14·1
	12·6	14·9
	14·2	18·8
	15·2	20·0
	19·1	21·1
	23·2	22·3
Mean	**12·4**	**14·9**
S.D.	±5·5	±5·6

in the diet. This confirmation of the statements in the literature may not be as unambiguous as it appears. The lipotropic effects observed in the rats of groups *TC*-2 and *TC*-3 may be artefacts, since fatty livers and haemorrhagic renal lesions do not develop unless an adequate intake of food is maintained. The reason for the decreasing food intake of these weanling rats consuming diets containing such relatively small amounts of TECl is not known. Certainly the decreasing food intake was not due to kidney failure, since these organs were normal; nor was it likely to be due to toxicity, since older rats can eat diets containing about four times as much as the highest dosage given these weanlings. There is a more acute need for choline *per se* as a structural element in younger rats than in older ones; failure of growth may possibly be the explanation for the apparent lipotropic properties of TECl when fed to weanling rats. The paired-feeding experiments required to settle this point will be undertaken shortly.

Table 4. *Comparison of* TECl *and choline chloride in weanling rats*

(Rats weighing 36–42 g. (average 39 g.) fed diet *T*45 supplemented as shown below. The number shown under the heading 'kidney lesions' is the number of animals showing grossly swollen and darkened ('haemorrhagic') kidneys.)

Group no.	Supplement (in terms of choline equiv.) (mg./10 g.)	Duration (days)	No. of rats	Change in body wt. (g.)	Daily food intake (g.)	Average liver wt.		Total liver lipids (mg.)	Kidney		
						Wet (g.)	Dry, fat-free (g.)		Wt. (g.)	Body wt. (%)	Lesions
TC-0	None (basal)	9	10	− 1	3·4	3·40	0·576	671	1·18	3·11	10
TC-1	TECl≡8	9	10	+ 1	3·0	2·72	0·489	462	0·89	2·22	2
TC-2	TECl≡16	14	10	+ 1	2·9	2·57	0·600	197	0·77	1·98	0
TC-3	TECl≡32	14	8	− 2	2·5	2·35	0·557	149	0·62	1·68	0
TC-4	Choline Cl≡8	14	5	+30	6·0	5·97	1·102	1264	1·05	1·50	0

consuming the hypolipotropic basal ration (*TC*-0) became moribund by the ninth day owing to the severely haemorrhagic condition of their kidneys. Coincidentally with the rapid development of the renal lesions between the fifth and ninth days, the food consumption dropped to almost nothing, the average intake on the first day was 5·7 g., on the last day it was only 1·2 g. From other experiments it is known that the liver lipids of these weanlings reaches a maximum (about 23% fresh liver weight) on the sixth day. As uraemia developed, and doubtless as a result of the decreasing food intake, the liver lipids decreased slightly to the value noted (19·7%) at the time of killing.

A marked decrease in liver lipids and in the incidence of haemorrhagic renal lesions was observed as the TECl content of the ration increased. In striking contrast to the rapid growth occasioned by the choline supplement (cf. group *TC*-4, in which the rats almost doubled their weight in 2 weeks) was the complete failure to grow when TECl was present

SUMMARY

1. Triethyl-2-hydroxyethylammonium chloride (TECl) possesses a low chronic oral toxicity as well as low acute oral toxicity; the LD_{50}, about 316 mg. for rats weighing 160 g., indicates that TECl is approximately twice as toxic as choline chloride when given orally.

2. Lipotropic dose-response curves for TECl and choline chloride are presented from which the lipotropic activity of TECl is seen to be about one-fifth of that of choline chloride (on a molecular basis).

3. Male rats did not exhibit any lack of lipotropic response to TECl, i.e. both sexes responded similarly.

4. Supplements of TECl added to hypolipotropic basal diets did not permit growth, but did prevent the development of haemorrhagic kidneys in weanling rats. The significance of this apparent confirmation of statements in the literature is discussed.

We are indebted to Prof. C. H. Best, F.R.S., for his encouragement and helpful criticism, and to Drs Jessie H. Ridout and Jean M. Patterson who determined the lipids and assisted in other ways. The expenses of this investigation were defrayed, in part, by a grant from the Banting Research Foundation.

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The Effect of Surface-active Agents on Bacterial Glutamic Decarboxylase and Glutaminase

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In previous papers (Krebs, 1948; Hughes, 1949) it was shown that *n*-hexadecyltrimethylammonium bromide (Cetavlon) accelerates the decarboxylation of glutamate and the deamidation of glutamine by intact cells and extracts of *Clostridium welchii*, *Proteus morgani* and *Escherichia coli*. Kinetic studies (Hughes, 1949) suggested that the effect of the detergent might be due to the removal of a competitive inhibitor. In the present work the effect of a variety of surface-active agents on the bacterial glutamic acid decarboxylase and glutaminase has been studied. Acceleration was given only by those cationic surface-active agents which can form micelles, and the concentration of the agents required to form micelles was about the same as that producing maximum acceleration.

MATERIAL AND METHODS

Organisms and extracts. Intact washed cells and extracts of *Cl. welchii*, *Pr. morganii* and *Esch. coli* were prepared as previously described (Hughes, 1949).

Glutamine. Crude L-glutamine (66% pure as judged by amide/N estimation) was prepared from mangel-wurzels by the procedure described by Vickery, Pucher & Clark (1935). It contained, among other impurities, 2% arginine and traces of Zn (see Hughes, 1948), and was freed from these by the following method (see Archibald, 1945). A solution of crude glutamine (35 g.) in 400 ml. water at 60° was cooled to room temperature, saturated with H_2S, and left for 2 hr. After addition of norite (4 g.) the suspension was filtered through paper on to a column (1·5 cm. diameter and 30 cm. long) of 60–80 mesh permutite (Permutite Co. Ltd., Gunnersbury Avenue, London, W. 4), which had been previously washed with 0·5% NaCl. The glutamine solution was allowed to run through by gravity, and the column was washed twice with 20 ml. water. The combined washings and filtrate were evaporated under reduced pressure at 40° until crystals appeared. After addition of warm ethanol (2 vol., at 40°), the solution was left at room temperature and then at 2° until crystallization had finished. The glutamine crystals were collected, recrystallized from water-ethanol as before, washed with 60% ethanol, 40% water (v/v) mixture and dried *in vacuo* over P_2O_5. Yield 22 g. (Found: amide N, 9·4; total N, 18·9; CO_2 evolved by the method of Gale (1947), 28·6; arginine, 0·01. Calc. for $C_5H_{10}O_3N_2$: amide N, 9·6; total N, 19·2; CO_2, 29·6%.)

n-*Alkyltrimethylammonium bromides.* The Cetavlon (*n*-hexadecyltrimethylammonium bromide) was a specially pure sample supplied by Mr S. Ellingworth, Imperial Chemical Industries (Pharmaceuticals) Ltd. Other homologues in this series were synthesized from the *n*-alkyl bromides by treatment with trimethylamine. The alkyl bromides were prepared from the corresponding alcohols which were obtained as follows: commercial samples of *n*-heptyl, *n*-decyl and *n*-dodecyl alcohols were supplied by L. Light and Co. Ltd., Wraysbury, Bucks, and *n*-octadecyl alcohol by British Drug Houses Ltd. These were purified by fractional distillation. Tetradecyl alcohol was prepared by the method of Levene & Taylor (1924) from recrystallized

and redistilled myristic acid (British Drug Houses Ltd.). Docosyl alcohol ($C_{22}H_{45}O$) was obtained from erucic acid, prepared from rape-seed oil. The acid was converted into the ethyl ester (m.p. 48–49°) (Levene & Taylor, 1924), which was recrystallized from ethanol and converted into docosyl alcohol according to Adam (1925). The yield of the latter (m.p. 83°) was 15% of the erucic acid used; the greatest loss occurred in the last step of preparation.

The alcohols were converted to the corresponding *n*-alkyl bromides by the method of Kamm & Marvel (1920). Refluxing the *n*-alkyl bromides in an excess of alcoholic trimethylamine (Scott & Tartar, 1943) resulted in the formation of the corresponding *n*-alkyltrimethylammonium bromides, which were recrystallized twice from acetone or acetone-benzene according to solubility. The yield of the conversion of *n*-alkyl bromides to the alkyltrimethylammonium bromides was 60–80%. The halogen content was estimated by Volhard's method and was correct within ±2%.

Measurement of enzyme activity. The rate of decarboxylation of glutamate and glutamine was determined as described previously (Hughes, 1949). The rates are expressed as Q_{CO_2} (μl./mg. dry wt. cells/hr.). When extracts were used, Q_{CO_2} referred to the dry wt. of cells from which the extracts had been prepared.

Chemical estimations. Amide N was determined by hydrolysis at pH 6·0 at 100° (Vickery, Pucher, Clark, Chibnall & Westall, 1935) or enzymically with suspensions of *Cl. welchii* (Krebs, 1948). Arginine was determined by estimation of the urea formed on the addition of arginase. Zn was estimated by the dithiozone method of Vallee & Gibson (1948).

RESULTS

The effect of n*-alkyltrimethylammonium bromides*

Concentration of Cetavlon and decarboxylation of L*-glutamate.* Cetavlon had no appreciable effect on the decarboxylation of glutamate by intact cells and extracts of *Cl. welchii* when the concentration was below 5×10^{-5}M. When the concentration was raised above 5×10^{-5}M the degree of acceleration rose sharply with increasing concentration and was maximal between 2·5 and $5{\cdot}0 \times 10^{-4}$M. The rate of decarboxylation was reduced approximately 10–15% by 10^{-2}M-Cetavlon, and this inhibition increased as Cetavlon concentration was raised above 10^{-2}M. When the rates of decarboxylation (Q_{CO_2}) were plotted against the negative log. of the Cetavlon concentration, sigmoid curves were obtained (see Table 1 and curve 'Hexadecyl', Fig. 1), resembling curves showing the effect of the concentration of surface-active agents on other properties of their solutions. For instance, the change in electrical conductivity shows the same rapid approach to maximal effect with increasing concentration (Scott & Tartar, 1943). A similar curve is found when surface tension is measured (Ralston, 1946). According to McBain (1944), the abrupt change in the properties of surface-active agents is connected with the formation of micelles.

Homologous n*-alkyltrimethylammonium bromides.* The concentration at which surface-active agents form micelles ('critical point') decreases with increasing chain length of the alkyl substituent. The measurements of electrical conductivity of Scott & Tartar (1943) indicate that in the *n*-alkyltrimethylammonium bromide series a minimum chain length of at least eight carbon atoms is necessary before micelle formation can occur. If the ability to form micelles is essential for the action of a quaternary salt on the glutamic decarboxylase, it would be expected that homologues with an alkyl chain of less than eight carbon atoms would have no effect.

Table 1. *Rate of decarboxylation of glutamate at varying concentrations of Cetavlon*

(Each flask contained 2·2 ml. solution (extract of *Cl. welchii* equivalent to 2 mg. dry wt. cells; 0·1M-acetate buffer, pH 4·1; 0·0045M-glutamate; Cetavlon solution 0·5 ml.). All concentrations are final concentrations. After completion of decarboxylation, 2 drops of 0·04% methyl orange were added.)

Concentration of Cetavlon (M × 10^{-5})	Q_{CO_2} (first 10 min.)	Colour of methyl orange*
250	306	Canary yellow
125	306	Canary yellow
50	306	Canary yellow
37·5	306	Yellow orange
18·8	300	Orange yellow
12·5	132	Orange
6·25	132	Orange
3·25	132	Orange
0·14	132	Orange
None	129	Orange

* See text, p. 233.

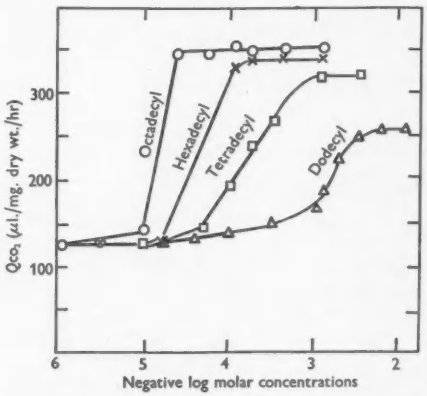

Fig. 1. Homologous *n*-alkyltrimethylammonium bromides and the rate of decarboxylation of glutamate by an extract of *Cl. welchii*. Each flask contained 2·2 ml. solution (extract equivalent to 3·5 mg. dry wt.; 0·1M-acetate buffer, pH 4·1; 0·0045M-glutamate; 0·5 ml. solution of the homologue, indicated in the figure). All concentrations are final concentrations.

Examination of a number of *n*-alkyltrimethylammonium bromides showed that this is the case. The ethyl and *n*-heptyl homologues did not accelerate the decarboxylation of glutamate by intact cells or extracts in concentrations up to 10^{-1}M, and were inhibitory at higher concentrations. The decyl homologue accelerated (20%) in all batches of intact cells, but only in some batches of extracts, at a concentration of 5×10^{-2}M, while homologues with alkyl chains of between twelve and twenty-two carbon atoms accelerated the enzymic reaction in all batches of cells and extracts. Fig. 1 shows the concentration and effect of some members of the series on the rate of decarboxylation of glutamate in an extract. Similar results were obtained with intact cells. It will be seen that as the length of the alkyl chain increased the maximum acceleration was greater and the concentration of the homologue required to give maximum acceleration was lower. Another point of interest is that the slope of the ascending parts of the curves was the steeper the longer the alkyl chain. The maximum effect was obtained by homologues with an alkyl chain of sixteen carbon atoms in the case of cells, and from eighteen to twenty carbon atoms in the case of extracts (see Table 2).

Table 2. *Maximum acceleration of the decarboxylation of glutamate by homologous* n*-alkyltrimethylammonium bromides*

(Each flask contained 2·2 ml. solution (extract or cells of *Cl. welchii* equivalent to 3·15 mg. dry wt.; 0·1M-acetate buffer, pH 4·1; 0·0045M-glutamate; 0·5 ml. solution of homologue). All concentrations are final concentrations.)

Homologue added	Minimum concentration of homologue causing maximum acceleration (M)	Q_{CO_2} (first 10 min.) Intact cells	Extract
None	—	85	65
Ethyl	2×10^{-1}	85	65
Heptyl	5×10^{-2}*	85	65
Decyl	5×10^{-2}	102	65
Dodecyl	3×10^{-2}	146	68
Tetradecyl	3×10^{-3}	149	100
Hexadecyl	4×10^{-4}	153	123
Octadecyl	8×10^{-5}	136	134
Docosyl	1×10^{-4}	130	134

* The heptyl homologue inhibited at 10^{-1}M.

These results further support the assumption that micelle formation is an important factor in the effect of Cetavlon on decarboxylation.

Detection of micelles by the use of indicators. Hartley (1923) showed that surface-active agents in concentrations above their critical points change the colour of certain indicator solutions although the pH remains unchanged. The colour change is presumably due to the dye being absorbed by, or dissolved in, the micelles of the detergent. Cetavlon and some other surface-active agents change the colour of solutions of methyl orange in buffer at pH 4·0 from orange to a bright canary yellow. When solutions of methyl orange in acetate buffer containing Cetavlon were compared in a Beckman spectrophotometer with solutions of the dye in anhydrous butanol, the adsorption curves of the solutions were almost identical and markedly different from the orange solutions of the dye in 0·1N-NaOH.

To test qualitatively for micelle formation, two drops of 0·4% aqueous methyl orange were added to the Warburg flasks after the completion of the incubation period. The flasks were then returned to the thermostat for 5 min. and the colour noted. The presence of a bright canary yellow colour was taken as an indication of the presence of micelles.

Corrin & Harkins (1946) used the colour change of indicators by surface-active agents to estimate the 'critical point' of micelle formation. To test the suitability of methyl orange for this estimation the homologous *n*-alkyltrimethylammonium bromides were titrated with the dye in the following manner. Methyl orange (10^{-4}M in 0·05M-acetate buffer, pH 4·1) was placed in a test tube in a comparator, and 2% Cetavlon was then added until the colour was bright yellow. This tube served as a colour control. To another tube, 2·0 ml. of the methyl-orange solution was added, and the solution of the homologue under examination added dropwise from a 2·0 ml. burette. The concentration of the homologue was adjusted to give a reading of from 1·0 to 1·5 ml. when the colour of the experimental tube matched that of the control tube. When the titrations were carried out quickly, in order to avoid the formation of a precipitate which developed on standing, the values for the critical point agreed to within ±5%. The results show approximate agreement with those obtained when 2:6-dichlorophenol-indophenol was used as in the method of Corrin & Harkins (1946) (Table 3). Both sets of results

Table 3. *Maximum acceleration of decarboxylation of glutamate and 'critical point' determined by titration with indicators*

(Maximum acceleration determined in experiments illustrated in Fig. 1; 'critical point' by titration with 2:6-dichlorophenolindophenol and with methyl orange as described in the text.)

n-Alkyltrimethylammonium bromide added	Concentration causing maximum acceleration (M)	'Critical point' (M) 2:6-Dichlorophenolindophenol	Methyl orange
Decyl	$5{\cdot}0$–$7{\cdot}0 \times 10^{-2}$	$6{\cdot}0 \times 10^{-2}$	$6{\cdot}0 \times 10^{-2}$
Dodecyl	$1{\cdot}0$–$3{\cdot}0 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	$2{\cdot}0 \times 10^{-2}$
Tetradecyl	$1{\cdot}0$–$3{\cdot}0 \times 10^{-3}$	$1{\cdot}0 \times 10^{-3}$	$2{\cdot}0 \times 10^{-3}$
Hexadecyl	$2{\cdot}0$–$5{\cdot}0 \times 10^{-4}$	$5{\cdot}0 \times 10^{-4}$	$3{\cdot}0 \times 10^{-4}$
Octadecyl	$6{\cdot}0$–$8{\cdot}0 \times 10^{-5}$	$1{\cdot}0 \times 10^{-4}$	$1{\cdot}0 \times 10^{-4}$

obtained by indicator methods tended to be lower than those obtained by conductimetric methods by Scott & Tartar (1943), probably owing to the effect of acetate on the critical point (Corrin & Harkins, 1947).

Micelles and the acceleration of decarboxylation. As shown in Table 1, concentrations of Cetavlon which change the colour of methyl orange from orange to bright yellow, indicating the presence of micelles, accelerated decarboxylation. On the other hand, when the colour of methyl orange remained unchanged the rate of decarboxylation was not accelerated. Under a variety of experimental conditions with intact cells and with extracts, the colour change of methyl orange and the acceleration of decarboxylation ran parallel. Similar results to that given by Cetavlon were given by other members of the homologous series with chain length of more than ten carbons. The ethyl and heptyl homologues formed no micelles according to the methyl-orange test, nor did they accelerate decarboxylation.

Methyl orange added to suspensions of intact cells was absorbed on to the cells. When added to solutions containing extracts, the dye was absorbed on to a precipitate formed from the extract. The colour of the absorbed dye appeared to be similar to that of the dye in solution; that is to say when no Cetavlon was present the colour of the dye was orange, and when Cetavlon micelles were present it was yellow. It would appear, therefore, that Cetavlon micelles are able to change the colour of dye absorbed on to enzyme material as well as that of dye in solution. The critical point of Cetavlon in acetate buffer alone was $5{\cdot}0 \times 10^{-4}$M, and in buffer with enzyme material it ranged from 2·0 to $5{\cdot}0 \times 10^{-4}$M. Thus enzyme material has no major effect on the critical point, although some Cetavlon is probably bound by the enzymic material. Maximum acceleration for the *n*-alkyltrimethylammonium bromide occurs approximately at the critical point as titrated with indicators (see Table 3). Experiments with other types of cationic detergents showed that maximum acceleration occurred only when methyl orange was changed to bright yellow colour, suggesting that with these compounds maximum acceleration and critical point also coincide.

The effect of compounds other than n*-alkyltrimethylammonium bromides*

Quaternary ammonium compounds. Betaine, trigonelline, choline and acetylcholine were examined at various concentrations up to 0·05M; they did not accelerate or inhibit the decarboxylation of glutamate and glutamine by intact cells and extracts of *Cl. welchii*.

Quaternary nitrogen surface-active agents. All substances tested in this series were found to accelerate the decarboxylation of glutamate and glutamine. The concentration giving the maximum rate of decarboxylation is shown in Table 4. Methyl-orange tests indicated the presence of micelles at the concentration producing maximum acceleration.

Cetavlon and *n*-hexadecylpyridinium bromide, which have the same alkyl substituent, give maximum acceleration at approximately the same concentration, but the degree of acceleration is greater in the case of the pyridinium compound. The effect of chain length of the alkyl group in the pyridinium series appears to be the same as in the alkyltrimethylammonium series.

Some of the other compounds listed in Table 4 give a greater acceleration than Cetavlon although the chain length of their alkyl substituent is shorter.

Other surface-active agents. Other cationic long-chain salts (Table 5) accelerate the decarboxylation of both glutamate and glutamine. The following amidines, not listed in Table 5, were found ineffective: *N'*-*p*-chlorophenyl-N^5:N^5-di-*n*-butyldiguanide acetate, *N'*-α-naphthyl-N^5:N^5-dimethyldiguanide hydrochloride, *N*-(2'-diethylaminoethyl)-2-naphthamidine dihydrochloride (and dihydro-

Table 4. *Quaternary nitrogen surface-active agents and the acceleration of decarboxylation*

(Each flask contained 2·2 ml. solution (intact cells 3·5 mg. dry wt.; 0·1M-acetate buffer, pH 4·1; substance to be tested, 0·5 ml.; 0·0045M-glutamate or glutamine). All concentrations are final concentrations.)

Detergent added	Approximate concentration of detergent causing maximum acceleration (M)	Q_{CO_2}	
		Glutamate	Glutamine
None	—	362	146
Cetavlon	5×10^{-4}	740	406
N-*n*-Dodecylpyridinium bromide	2×10^{-3}	815	435
N-*n*-Hexadecylpyridinium bromide	2×10^{-4}	900	540
N-*n*-Dodecoxymethylpyridinium dithionite	5×10^{-4}	750	368
n-Dodecyl ester of triethylcarboxymethylammonium chloride	2×10^{-4}	865	420
2-(4:6-diaminopyrimidino)-methyldimethyl-*n*-dodecylammonium bromide	1×10^{-3}	805	480
n-Hexadecyl ester of *p*-carboxy-*N*:*N*:*N*-trimethylanilinium hydrogen sulphite	4×10^{-4}	685	485

Table 5. *Non-quaternary surface-active agents and the acceleration of decarboxylation*

(Each flask contained 2·2 ml. (intact cells 3·5 mg. dry wt.; 0·1M-acetate buffer, pH 4·1; substance to be tested in 0·5 ml.; 0·0045M-glutamate or glutamine). All concentrations are final concentrations.)

Compound added	Approximate concentration for maximum effect (M)	Q_{CO_2}	
		Glutamate	Glutamine
None	—	362	146
Cetavlon	5×10^{-4}	740	406
Ethanolamine ester of dodecane-1-carboxylic acid (hydrochloride)	3×10^{-3}	990	359
Ethanolamine ester of stearic acid (hydrochloride)	$2{\cdot}6 \times 10^{-4}$	1005	540
Stearamidine hydrochloride	4×10^{-4}	900	460
Ethyl-*n*-hexadecylamine	1×10^{-3}	750	406
Methyldihexylamine	$2{\cdot}5 \times 10^{-2}$	190	200
Methyl-*n*-octylamine	8×10^{-2}	740	406

bromide). Active compounds showed the methyl-orange reaction for micelles but inactive compounds did not.

Anionic surface-active agents. Sodium dodecyl sulphate and dioctyl sodium sulphosuccinate (Aerosol OT) were found to be powerful inhibitors of the decarboxylation of glutamate and glutamine, at concentrations above (10^{-2}M) and below (10^{-5}M) their critical points. Ammonia formation from glutamine was inhibited at concentrations below the inhibitory range for the decarboxylation of glutamate.

Non-ionic surface active agents. Tween 80 and Tween 20 (described by the makers, Atlas Powder Co. London, as 'polyoxyalkalene sorbitans') were purified by the method of Archibald (1946). They did not accelerate decarboxylation of glutamate or of glutamine by extracts of *Cl. welchii*, *Pr. morganii* and *Esch. coli*; they slightly accelerated (20%) the decarboxylation of glutamine, but not glutamate, by intact cells of *Cl. welchii* and *Pr. morganii*. This effect was not investigated further. Both compounds changed the colour of methyl orange to a bright yellow colour indicating the presence of micelles under the conditions of test.

n-*Fatty acids.* Solutions of the sodium salts of butyric, valeric and pentane-1-carboxylic acids were adjusted to pH 6·0 with acetic acid. These solutions did not appreciably alter the pH of the decarboxylation system. Higher fatty acids prepared in this way altered the pH of the decarboxylation system because of the low solubility of the free acid, e.g. 0·5 ml. of 0·1M-heptane-1-carboxylate added to 1·0 ml. of 0·2M-acetate buffer at pH 4·0 changed the pH to 4·3. In order to prevent this change in pH, heptane-1-carboxylic, octane-1-carboxylic, nonane-1-carboxylic, lauric, palmitic and oleic acids were emulsified in 0·2M-acetate buffer, pH 4·0. These emulsions, when added to the decarboxylation system in quantities sufficient to give a concentration of acid of 3×10^{-2}M, did not appreciably change the pH.

Butyric, valeric and pentane-1-carboxylic, acids had no effect on the rate of decarboxylation of glutamate or glutamine in intact cells of *Cl. welchii*. Heptane-1-carboxylic, octane-1-carboxylic and nonane-1-carboxylic acids slightly (10–15%) accelerated the decarboxylation of both substrates in intact cells. In cell-free extracts fatty acids had no effect.

DISCUSSION

A parallelism has been established between the capacity of cationic surface-active agents to accelerate the decarboxylation of glutamate and glutamine by *Cl. welchii* and other bacteria on the one hand, and the capacity to form micelles on the other. It was suggested in a previous paper (Hughes, 1949) that Cetavlon acts by removing a competitive inhibitor accompanying the enzymes in cells and extracts, either by solubilization or by chemical complex formation. The experiments reported in this paper show that anionic and non-ionic detergents do not accelerate the enzymic reactions although they form micelles. Thus the charge on the micelle is an essential factor in the activity of the detergents, and this favours the view that the inhibitor is removed by complex formation. The nature of such complexes has been reviewed by Putman (1948).

The presence of an inhibitor together with the enzymes is not improbable, as both glutamate and glutamine take part in other enzymic reactions for which they must to some extent be protected against the action of the decarboxylase and glutaminase.

SUMMARY

1. An acceleration of the decarboxylation of glutamate by extracts and intact cells of *Clostridium welchii*, previously reported for Cetavlon, is given by homologous *n*-alkyltrimethylammonium bromides. The minimum number of carbon atoms in the alkyl substituent needed to give the effect is ten for both

intact cells and extracts. The number of carbon atoms causing maximum acceleration is sixteen in the case of cells and from sixteen to eighteen in the case of extracts.

2. A parallelism was observed between the ability of cationic detergents to form micelles in aqueous solution, and their ability to accelerate the decarboxylation of glutamate and glutamine. Maximum acceleration of the decarboxylase within the homologous series approximately coincided with the 'critical point of micelle formation' as determined by the colour change of indicators.

3. Anionic surface-active agents inhibited the decarboxylation of glutamate and glutamine. Non-ionic surface-active agents and fatty acids were without effect.

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Studies in Detoxication

30. THE METABOLISM OF BENZENE. (*a*) THE DETERMINATION OF BENZENE. (*b*) THE ELIMINATION OF UNCHANGED BENZENE BY RABBITS

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In general, the metabolism of benzene proceeds along four paths, namely, (1) oxidation to phenols, (2) elimination unchanged, (3) formation of phenylmercapturic acid, and (4) ring fission to muconic acid and other products. The first of these was quantitatively studied by Porteous & Williams (1949), who concluded that in the rabbit an average of 21% or, at most, about 30% of an oral dose of benzene at the level of 500 mg./kg. was metabolized to phenols. At least 70% of the administered benzene must take other paths. The present work deals with the second path, the elimination unchanged. It is found that at a level of 500 mg./kg. about 40% of orally administered benzene is eliminated unchanged through the lungs. Thus the first two paths can account for at least 60% or, taking the higher values of some experiments, up to 80–90% of the dose.

That orally administered benzene might be excreted unchanged through the lungs was first suggested by Schultzen & Naunyn (1867), but Munk (1876) doubted this possibility. In 1883, Nencki & Sieber, after experiments on men, dogs and rabbits, expressed the view that approximately one-third of benzene was excreted as phenol conjugates, one-third as catechol and quinol and the remaining third must come out unchanged. The first quantitative experiments on the elimination of unchanged benzene were carried out by Lehmann, Stohr, Kleiner & Gundermann (1910). Tracheotomized rabbits were allowed to inhale benzene-air mixtures, and from an analysis of the inspired and expired air, the amount of benzene absorbed was ascertained. During 1 hr. after benzene ceased to be inhaled, 25% of the absorbed benzene was exhaled unchanged. When the period was increased to 4 hr. then 34% was exhaled,

but elimination over longer periods was not studied. In man, 6% was exhaled during 15 min. after ceasing to inhale benzene. Peronnet (1935) found that guinea pigs inhaling benzene excreted 4 mg. through the lungs in 5 min., but the amount of benzene inspired was not known. There appears to be only one observation on the elimination of free benzene through the kidneys. Drummond & Finar (1938) stated that free benzene can be detected in the urine of rabbits only when the dose exceeds 3 ml.

Thus the only relevant determinations of the elimination of free benzene are those of Lehmann *et al.* (1910), but unfortunately their longest experiments only lasted 4 hr. and were concluded whilst excretion was at the appreciable rate of 3% of the dose/hr. Our experiments were carried out until only traces of benzene were found in the expired air and the benzene was administered in a more controlled manner by the oral or parenteral routes.

EXPERIMENTAL

A. *The determination of benzene*

Of several methods available for the determination of benzene the method of Schrenk, Pearce & Yant (1935) is very sensitive, and we directed our attention to this.

Peltzer (1933) described colour reactions between *m*-dinitrobenzene and ketones in the presence of concentrated alkalis, and from these Pearce, Schrenk & Yant (1936) developed a micromethod for the determination of benzene in blood and urine. In this method benzene is blown in a stream of air into a mixture of concentrated nitric and sulphuric acids, and the *m*-dinitrobenzene formed is then extracted from the neutralized mixture with methyl ethyl ketone. A purple colour is then developed by adding aqueous 40% (w/v) sodium hydroxide. Baernstein (1943), Alekseeva (1940) and Bykhovskaya (1945) described modifications of the method.

The method of Schrenk *et al.* (1935) is sensitive to 1 μg. of benzene with an accuracy of 10%. However, a long time (1·5 hr.) is necessary for development of the colour, which is produced in a two-phase system. We have now investigated the method in detail and studied aspects not appreciated by earlier investigators. An analytical procedure has resulted in which maximum colour production is almost instantaneous in a one-phase system without loss of accuracy or sensitivity. Our investigations have covered the influence of the nature of the ketone and of the alkali, the concentration of ethanol and of water, and the presence of *o*- and *p*-dinitrobenzene upon the production of the colour.

The mechanism of colour development, discussed by Baernstein (1943), is that, in the presence of alkali, the acid forms of certain nitro compounds condense with the enol forms of ketones to form coloured quinonoid structures.

(1) *The choice of ketone.* In order to extract *m*-dinitrobenzene from the neutralized nitrating mixture, whilst avoiding such solvents as ether, a ketone incompletely miscible with water was necessary. With aliphatic ketones it was found that the intensity of the colour diminished with increasing number of C atoms, and we were immediately limited to methyl ethyl ketone and *cyclo*hexanone. Since the colour developed in *cyclo*hexanone was much more sensitive to alkali concentration than that in methyl ethyl ketone (Fig. 1), the latter ketone became the obvious choice.

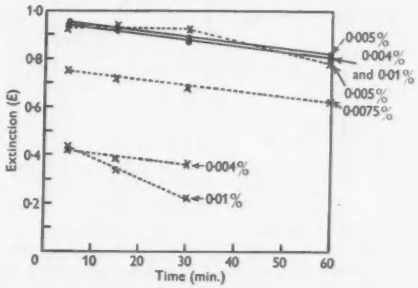

Fig. 1. The influence of alkali concentration on the stability of the colour formed between *m*-dinitrobenzene and methyl ethyl ketone or *cyclo*hexanone. The data for this figure was obtained by taking 2 ml. of *m*-dinitrobenzene solution (40 μg./ml.) in methyl ethyl ketone or *cyclo*hexanone and adding 0·1 ml. of ethanolic NaOH (concentration 0·4, 0·5, 0·75 and 1% (w/v)) and making up to 10 ml. with the ketone. Readings were taken at intervals with the Spekker absorptiometer. Final alkali concentrations are given on the figure. Precipitation occurred in some of the *cyclo*hexanone solutions. Full lines, methyl ethyl ketone; broken lines, *cyclo*hexanone.

(2) *The nature and concentration of the alkali.* The strong organic bases piperidine and benzylamine gave no colour with *m*-dinitrobenzene in any of the ketones tested. Na ethoxide in dry ethanol gave an unstable violet solution which rapidly turned brown owing to reaction between the ketone and ethoxide. In order to avoid obtaining a two-phase system which results from the use of aqueous alkali we tried ethanolic NaOH and KOH. In general, it was found that the colour was more stable with NaOH than with KOH. In methyl ethyl ketone the colour is completely destroyed in less than 5 min. if the alkali concentration is greater than 0·5%. Between 0·05 and 0·5%, there may be precipitation of alkali. Below 0·002% alkali the full colour is not developed. Some of our results are shown in Fig. 2 which again shows the difference between *cyclo*hexanone and methyl ethyl ketone. The concentration of alkali finally adopted was 0·005% NaOH.

(3) *The effect of ethanol.* The stability of the colour is also affected by ethanol, some of which had to be used because the best alkali reagent found was ethanolic NaOH and some ethanol was necessary to maintain a homogeneous system. The effect of ethanol concentration is shown in Fig. 3. It was found that if the ethanol concentration is much below 1%

precipitation of the alkali occurred, whereas if it exceeds 2% the maximum development of colour is impaired. Methanol can be substituted for ethanol.

(4) *Effect of water.* Since, in the determination of benzene, the *m*-dinitrobenzene formed by nitration has to be extracted with methyl ethyl ketone from an aqueous solution, the influence of the concentration of water on the development of the colour had to be investigated. The results are summarized in Fig. 4. Water in a concentration up to 0·5% has no influence on the colour. Above this concentration, the colour is slower to develop and there is a loss of intensity. Since the solubility of water in methyl ethyl ketone is 10%, the original ketone extract must be diluted at least 1 : 20 with ketone prior to colour development to ensure that the concentration of water is less than 0·5%.

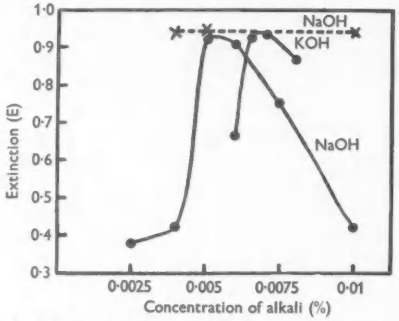

Fig. 2. Effect of NaOH or KOH concentration on the intensity of the colour from *m*-dinitrobenzene and methyl ethyl ketone (broken line) or *cyclo*hexanone (full lines). Solutions prepared as for Fig. 1 and read 5 min. after mixing.

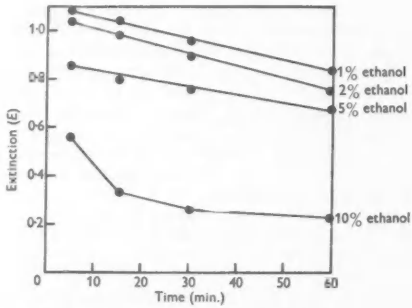

Fig. 3. Effect of ethanol concentration. The curves were obtained by adding to 1 ml. of *m*-dinitrobenzene in methyl ethyl ketone (100 μg./ml.), 0·1 ml. of 1%, 0·2 ml. of 0·5%, 0·5 ml. of 0·2% and 1 ml. of 0·1% ethanolic NaOH respectively and then making up to 10 ml. with the ketone. The colour was then read in the absorptiometer at intervals. Ethanol concentrations are given on the figure. The alkali concentration was constant throughout.

(5) *The influence of* o- *and* p-*dinitrobenzene.* In the present work the nitration of benzene was carried out at room temperature (18–25°) with conc. H_2SO_4 and fuming HNO_3, and significant amounts of *o*- and *p*-dinitrobenzene are formed. From the work of Wyler (1931), it appears that about 93–94% of *m*-, 4–5% of *o*- and 2·0–2·5% of *p*-dinitrobenzene are produced under our conditions. We therefore investigated the colour produced by the latter two isomers with alkali in the presence of methyl ethyl ketone. Pure samples of each were prepared (*o*-, m.p. 116·5° and *p*-, m.p. 174°). Solutions of the three isomers were made up in various concentrations in methyl ethyl ketone, by adding 0·5–5·0 ml.

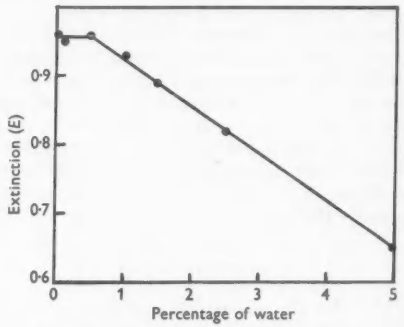

Fig. 4. Effect of water concentration. Varying amounts of a 10% solution of water in methyl ethyl ketone were added to 2 ml. of *m*-dinitrobenzene (40 μg./ml.) in the ketone. The colour was developed with 1 ml. of 0·05% ethanolic NaOH, volume made up to 10 ml. with the ketone and readings taken after 5 min.

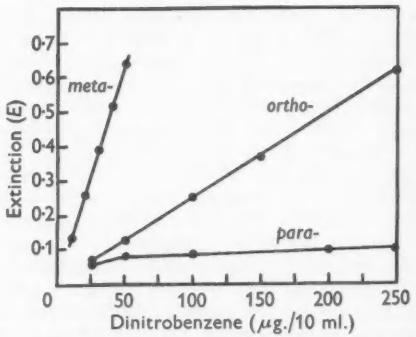

Fig. 5. A comparison of the colour intensities produced by the action of ethanolic NaOH on solutions of *o*-, *m*- or *p*-dinitrobenzene in methyl ethyl ketone.

of a 50 μg./ml. standard solution to 10 ml. flasks and diluting with the ketone. The colour was developed by adding 1 ml. of the 0·05% 'alkali reagent' (see below) and making up to 10 ml. with the ketone. The colours were read, after developing 5 min. in the dark, in a Spekker absorptiometer using a yellow-green filter (Ilford 605) and the heat filter (no. H 503). A blank was made up by diluting 1 ml. of the alkali reagent to 10 ml. with methyl ethyl ketone. The *o*-isomer gave a blue colour. The *p*-isomer also gave a blue colour which rapidly faded in 2–3 min. The relative intensities of the colours are shown in Fig. 5.

From this graph it can be calculated that, in the concentrations likely to be encountered in this work, the 4–5% of the *ortho* isomer present in the nitrated benzene mixture will contribute a colour equivalent to approximately 1% of *m*-dinitrobenzene. The 2% of *para* isomer present will contribute an equivalent of only 0·2% of *m*-dinitrobenzene. It is therefore clear that the maximum amount of benzene recoverable as *m*-dinitrobenzene using this colour reaction is 94–95%. In direct nitration experiments we obtained maximum recoveries of nearly 95% (Table 1). All values for recovery of benzene were, therefore, corrected for the formation of *o*- and *p*-dinitrobenzene by multiplying the values found by 100/95. It should be noted from Table 1 that recovery of benzene by passage with a stream of air is better than by direct addition. There is little doubt that some benzene is lost as a result of its volatility when it is added directly to the nitrating mixture unless great care is taken.

Table 1. *The recovery of benzene added directly to a nitrating mixture*

(Method *A*. Benzene slowly added to 20 ml. of nitrating mixture at 20°. The dinitrobenzenes were extracted from the neutralized mixture with methyl ethyl ketone and estimated colorimetrically as described in the text. Method *B*. Benzene aerated from a 250 ml. Dreschel bottle into the nitrating mixture at 20° during 4 hr.)

		Benzene recovered		
Method	Benzene added (mg.)	(mg.)	(%)	Corrected for *o*- and *p*-dinitrobenzene (%)
A	465	435	93·5	98·4
A	455	415	91·3	96·1
A	328	308	93·9	98·8
A	328	305	93·0	97·9
A	492	466	94·7	99·7
B	953	902	94·6	99·6
B	937	882	94·1	99·1

Construction of the standard curve for m-*dinitrobenzene*

Reagents. Methyl ethyl ketone was dried over anhydrous $CaCl_2$ and distilled, the fraction of b.p. 79–80° being used.

m-*Dinitrobenzene.* A commercial sample, m.p. 75–78°, was twice recrystallized from ethanol. This material formed long white needles, m.p. 89°. A stock 1% (w/v) solution in methyl ethyl ketone appeared to keep indefinitely. Standard solutions were prepared by diluting this.

Benzene. Thiophene-free benzene was dried over anhydrous $CaCl_2$ and distilled, and the fraction of b.p. 80–81° used.

The alkali reagent. The stock alkali solution was prepared by dissolving 0·5 g. of NaOH (A.R.) in absolute ethanol, filtering and making up to 100 ml. with ethanol. The 0·05% 'alkali reagent' was freshly prepared for each experiment by diluting the stock solution (filtered if necessary) with 9 vol. of methyl ethyl ketone. This reagent deteriorates in a few hours, turning brown and thus producing a high blank.

The nitrating mixture consisted of equal volumes of conc. H_2SO_4 (A.R.) and fuming HNO_3 (sp.gr. 1·5; A.R.).

Concentrated alkali. For neutralizing the nitrating mixture a 40% (w/v) aqueous solution of NaOH (B.P.) was used.

The standard curve (Fig. 6) for *m*-dinitrobenzene was constructed by adding to a series of 10 ml. graduated flasks 1–8 ml. of a 10 μg./ml. solution of *m*-dinitrobenzene in methyl ethyl ketone. These solutions were diluted to nearly 9 ml. with methyl ethyl ketone and then treated with 1 ml. of the 0·05% 'alkali reagent'. After making up to 10 ml. with

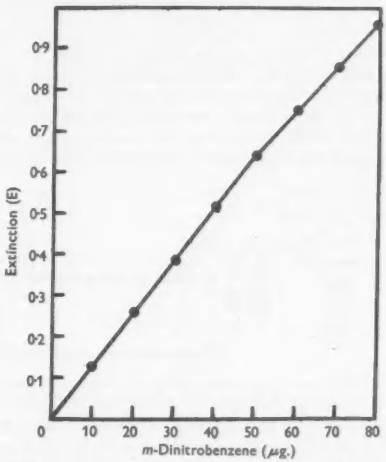

Fig. 6. Calibration curve for *m*-dinitrobenzene. Constructed as described in the text, p. 239.

the ketone and mixing, the colour was read, after 5 min. standing, in the Spekker absorptiometer using the colour filter Ilford no. 605 and heat filter no. H 503 and a 1 cm. cell. A standard blank was prepared by mixing 1 ml. 'alkali reagent' with 9 ml. methyl ethyl ketone. The curve obtained is linear from 0 to 40 μg./10 ml. and approximately linear up to 80 μg./10 ml.

Recovery of m-*dinitrobenzene from the nitrating mixture*

Various amounts of pure *m*-dinitrobenzene were added to 20 ml. of the nitrating mixture and left for 2 hr. The mixture was diluted with an equal volume of water and

Table 2. *Recovery of added* m-*dinitrobenzene from a nitrating mixture*

	m-Dinitrobenzene	
Added to 20 ml. of nitrating mixture (mg.)	Recovered (mg.)	(%)
20	19·8	99
20	20·0	100
50	50·1	100·2
50	49·9	99·8
100	100·2	100·2
500	502·0	100·4

neutralized with the 40% aqueous NaOH, the temperature being kept below 30° under the tap. A few drops of the alkali were then added in excess. The mixture, which had a volume of about 100 ml., was extracted with 2 × 15 ml. and then

2 × 10 ml. of methyl ethyl ketone and the extract made up to 50 ml. Suitable portions were then taken and the colours developed with the 'alkali reagent' as above. The recovery of *m*-dinitrobenzene in the range 20–500 mg. was accurate to 1% (Table 2).

B. *Experiments on the elimination of unchanged benzene*

Animals. Chinchilla rabbits, 2–3 kg. in weight, were used.

The animal chamber (Fig. 7) consisted of a Perspex tank of dimensions 60·5 × 33·5 × 22·5 cm. with an internal volume of 45·6 l. The lid of the tank was removable and was secured by twelve wing-nuts to the tank walls with a rubber diaphragm in between to ensure airtightness. This diaphragm

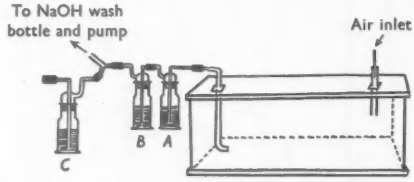

Fig. 7. Diagram of repiration tank and absorption train (see text, p. 240 for description). *A*, *B* and *C*, Dreschel bottles containing nitrating mixture. *C* is the control bottle which shows presence or absence of benzene in the atmosphere.

was painted with collodion so that no rubber surfaces were exposed to benzene. The lid was also equipped with three glass tubes fitted with ground glass stoppers lubricated with silicone high vacuum grease which is inert to benzene, and two copper air tubes, all these being affixed to the lid in rubber cushions whose exposed surfaces were painted with collodion. The tank also had a removable, perforated aluminium floor, enabling the animals to sit out of contact with any urine excreted during the experiment. To increase the draught at the air inlet and produce a current of air across the tank, air was admitted through a 3 mm. glass tube fitted into the glass tube diagonally opposite the copper outlet tube. The other copper air tube was sealed.

The absorption train (Fig. 7). This consisted of two 50 ml. Dreschel gas bottles in series with ground glass heads each containing 20 ml. of 2 mm. glass beads and 20 ml. of nitrating mixture. A third bottle, similarly filled, was connected in parallel and was used to check the in-going air for benzene. The three bottles were connected by a glass Y-piece to a wash-bottle containing 20% (w/v) aqueous NaOH to remove acid vapours and thence to the pump. To prevent loss of benzene through connexions, the latter were made of 'Portex' polyvinyl plastic tubing, which also resisted attack by nitrous fumes. All ground joints were lubricated with silicone high vacuum grease which is not permeable to benzene.

During prolonged aeration, the nitrating mixture becomes depleted of the oxides of nitrogen and may become so weakened as to produce only mononitrobenzene. In all experiments, therefore, the Dreschel bottles were changed every 2 hr. by fresh ones containing a new charge of beads and nitrating mixture.

The recovery of benzene from the tank. Known amounts of benzene in a weighing bottle (attached to a thread for tilting inside the tank) were introduced into the tank and the lid secured and connected to the absorption train. The benzene in the bottle was scattered inside the tank by pulling the attached thread, the end of which protruded through one of the orifices in the lid. The tank was now aerated at the rate of 20 l./hr. (measured with a flowmeter) until the benzene concentration reached a negligible value. This required approx. 12 hr. Since the capacity of the tank is about 45 l., aeration at 20 l./hr. removes 40% of the original atmosphere in 1 hr. and 99–100% in about 12 hr.

The contents of the Dreschel bottles were worked up as follows. After disconnecting they were allowed to stand for 1 hr. The contents were then poured into a beaker and the nitrating mixture decanted from the beads into a conical flask. The Dreschel bottle and head were repeatedly rinsed with water, the washings being added to the beads in the beaker to rinse them as well. The washings were now added to the nitrating mixture, the temperature being kept below 30°. The bottle and beads were finally rinsed with small volumes of 40% (w/v) aqueous NaOH which were added to the nitrating mixture with cooling. The mixture was now neutralized with 40% (w/v) NaOH and a few drops of the latter were added in excess. The final volume of the solution was about 100 ml. This was extracted (at 25–30° to prevent precipitation of salts) with 2 × 15 ml. and 2 × 10 ml. methyl ethyl ketone and the extract made up to 50 ml. Suitable portions were then taken and the colour developed as above.

Table 3 shows that 1 or 0·1 g. can be recovered almost quantitatively by aeration from the tank. The last figure in this table was obtained by adding benzene to the tank when it contained the warm intact body of a freshly killed rabbit. This experiment was carried out because it was possible that a considerable part of the exhaled benzene might be absorbed

Table 3. *Recovery of benzene from the respiration chamber*

(Temperature of nitrating mixture, 20–25°.)

Benzene in chamber (g.)	Rate of aeration (l./hr.)	Duration of aeration (hr.)	Benzene recovered (g.)	Benzene recovered (%)	Benzene recovered, corrected for *o*- and *p*- isomers (%)
1·023	20	14	0·967	94·5	99·5
0·989	20	14	0·936	94·6	99·6
0·958	30	10	0·913	95·3	100·3
0·1024	30	10	0·0965	94·2	99·2
0·0962	15	18	0·0905	93·9	98·9
0·0178	20	10	0·0152	85·5	89·9
0·946*	20	14	0·857	90·6	95·3

* With a freshly killed rabbit in the chamber (see text, above).

Table 4. *The elimination of unchanged benzene in the expired air of rabbits receiving benzene orally*

Rabbit no.	Dose (mg./kg.)	Duration of experiment (hr.)	Percentage of dose recovered (corrected for *o*- and *p*-dinitrobenzene)	Average (%)	Benzene catabolized (by difference) (%)	Benzene catabolized (by difference) (mg./kg.)
8	250	16	27·6	37·3	62·7	157
10	250	18	45·8			
12	250	20	38·5			
1	500	30	38·7	39·1	60·9	305
8	500	26, 22	31·7, 40·4			
9	500	28	43·1			
10	500	22	55·9			
11	500	30	37·8			
12	500	24, 22	39·1, 26·3			
8	500*	30	33·6	31·0	69·0	345
12	500*	30	28·2			
8	1000	28	56·1	64·3	35·7	357
10	1000	28	75·1			
12	1000	30	61·7			

* Injected.

by the fur of the animal and re-absorbed through the skin (cf. Lazarev, Brusilovskaya & Lavrov, 1931). It is clear from this experiment that, although recovery is a little less when the dead animal was present, the error introduced is only a small one.

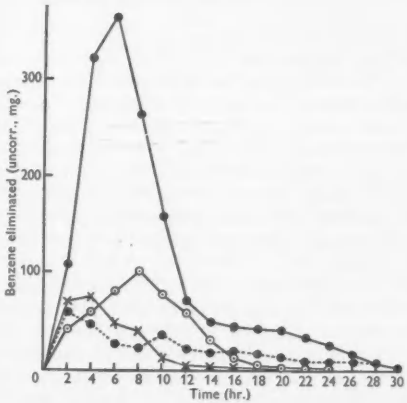

Fig. 8. The elimination in the unchanged state of ingested and injected benzene by rabbit no. 12. The actual amount of benzene eliminated (uncorrected) in each 2 hr. period is plotted against time. ●——●, 1 g./kg. orally; ⊙——⊙, 0·5 g./kg. orally; ×——×, 0·25 g./kg. orally; ●- - - -●, 0·5 g./kg. injected.

The blank was always negligibly low, being less than 0·5 mg./2 hr. aeration. Benzene and toluene were not used in the laboratory during an experiment. On one occasion a bench nearby was being polished with a mixture containing toluene and on this occasion the blank reached the equivalent of 1 mg. of benzene.

Elimination of ingested and injected benzene by rabbits

The experiments were carried out at three dose levels, 0·25, 0·5 and 1·0 g./kg. The rabbits were kept on a constant diet of 200 g. each of bran and cabbage a day prior to the experiments. For oral administration, the benzene was given suspended in 50 ml. water by a stomach tube made of 'Portex' polyvinyl plastic which does not absorb benzene

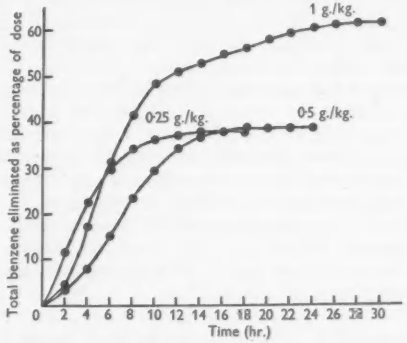

Fig. 9. The elimination in the unchanged state of orally administered benzene by rabbit no. 12. Total elimination, as percentage of dose (corrected), plotted against time.

as does rubber tubing. In the injection experiments only one dose level (0·5 g./kg.) was used and the benzene was injected intraperitoneally. Immediately after administration of benzene the rabbits were placed in the tank which was sealed and connected with the absorption train. The first absorption bottle was removed every 2 hr. and the contents analysed for *m*-dinitrobenzene, until only 0·1 % of the dose was found during a 2 hr. period. Most experiments lasted 20–30 hr., and at the end the contents of the second absorption bottle

were analysed for dinitrobenzene. The second bottle rarely contained more than 0·2% of the dose of benzene. The results are summarized in Table 4 and in Figs. 8 and 9, the results for rabbit no. 12 are expressed graphically. It is to be noted that the elimination of unchanged benzene takes longer when it is injected than when fed by mouth.

Excretion of unchanged benzene in the urine

First we required to know whether benzene added to urine could be recovered by aeration. Accordingly, 165 mg. of benzene were added to 100 ml. freshly voided rabbit urine in a Dreschel bottle and thoroughly mixed. A current of air was then passed for 6 hr. at 20 l./hr. through the urine into the absorption circuit and the *m*-dinitrobenzene estimated as before. The benzene recovered was 147 mg. or 94% (corrected value).

It was noticed on some occasions that when a rabbit in the tank voided urine there was a jump in the benzene elimination. This suggested that some unchanged benzene appears in the urine. Rabbits were therefore given 500 mg./kg. of benzene orally, placed in metabolism cages and the urine collected in Dreschel bottles placed beneath the funnels supporting the cage. As soon as urine was passed its benzene content was estimated by aeration. It was found that at this dose level no unchanged benzene appeared in the urine.

DISCUSSION

The elimination of unchanged benzene by the rabbit at a dose level of 500 mg./kg. appears to be entirely via the lungs, no detectable benzene appearing in the urine. The elimination at different dose levels is summarized in Table 4. Since the animals were kept in an enclosed chamber it is possible that our estimates of the unchanged benzene are slightly low, because the animal will rebreathe and absorb some of the benzene it has exhaled. At the dose level of 500 mg./kg. the average concentration of benzene in the chamber is about 4 mg./l. at the period of maximum excretion. However, basing our calculations on the experimental findings of Lehmann *et al.* (1910), we have assessed the amount of benzene reabsorbed during an experiment as being not more than 5–10 mg., despite the fact that during maximum excretion the total amount of benzene breathed in would be 120–140 mg./hr. Our results on this basis would not be more than 0·5% low.

The striking feature of Table 4 is that at the high dose level of 1 g./kg. a much greater percentage of the dose is eliminated unchanged than at the lower levels. This indicates that there is a limit to the amount of benzene which the rabbit can metabolize. This shows more clearly in the last column of Table 4. The rabbit therefore deals with benzene by eliminating it unchanged and by catabolizing it, i.e. by oxidation. The second way is, however, quantitatively limited, and it appears from Table 4 that it does not exceed about 300–350 mg. benzene/kg. of rabbit in about 24 hr. Thus up to 500 mg./kg. about two-fifths of the benzene administered comes out unchanged and three-fifths is catabolized. At the higher dose level of 1 g./kg. the animal still catabolizes benzene at about 350 mg./kg. but about two-thirds is eliminated unchanged and one-third is catabolized. Thus, on average figures, 11·2% of benzene at 500 mg./kg. is oxidized and excreted as glucuronides, 9·5% as ethereal sulphates (Porteous & Williams, 1949) and 39·1% is eliminated unchanged. On these figures some 60% of the benzene administered can be accounted for. It is felt that glucuronides, ethereal sulphates and unchanged benzene account for most of the benzene fed to rabbits, and, if all the estimations of these metabolites could be carried out simultaneously on the same animals, we have little doubt that they would account for 80–90% of the benzene administered. The problem of getting a balance sheet for the metabolism of benzene is, however, one of great difficulty owing to the scatter of the results; the extreme and average values obtained so far are as follows:

	(%)	Average (%)
As glucuronides	4·2–22·7	(11·2)
As ethereal sulphates	5·9–16·8	(9·5)
As phenol	6·3–17·0	(9·2)
Unchanged benzene	26·0–56·0	(39·1)

Other aspects of the metabolism of benzene, such as the formation of muconic acid and phenylmercapturic acid, are being actively pursued.

SUMMARY

1. The method for the determination of benzene based on nitration to *m*-dinitrobenzene and development of a colour by the interaction of the dinitrobenzene and a ketone in the presence of alkali has been carefully investigated. The influence of alkali, ethanol, water, nature of the ketone and *o*- and *p*-dinitrobenzene on colour development has been studied, and a rapid and improved method has been developed.

2. The elimination of unchanged benzene by rabbits receiving benzene by mouth and by injection has been investigated. By placing rabbits, after receiving benzene, in a respiration chamber, the amount of unchanged benzene eliminated was estimated by aeration into a nitrating mixture and subsequently determining the *m*-dinitrobenzene produced.

3. The benzene was eliminated during 15–30 hr., about 40% being recovered unchanged at the dose levels of 0·25 and 0·5 g./kg. and 64% at 1 g./kg.

4. The results have been discussed in relation to earlier studies on benzene and it has been shown that the ability of the rabbit to catabolize benzene is limited to roughly 300–400 mg./kg./day.

5. The benzene eliminated unchanged plus that oxidized and excreted as oxygen-conjugates accounts for at least 60% of the administered benzene.

We wish to express our thanks to Prof. J. M. Peterson and Dr John Pryde of University College, Cardiff, for the loan of the Perspex respiration tank used in this work. We are also grateful to Prof. Sir Alexander Fleming, F.R.S., for animal accommodation, and to Prof. A. St G. Huggett for the loan of a flowmeter.

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Studies in Detoxication

31. THE ISOLATION OF *m*- AND *p*-CYANOPHENOLS AS METABOLITES OF CYANOBENZENE (BENZONITRILE) AND THE PROBLEM OF THE ORIENTATION OF HYDROXYL GROUPS FORMED *IN VIVO*

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When monosubstituted benzenes carrying the classical *ortho-para*-directing groups (e.g. NH_2, NHAc, Cl, OH) are fed to animals, it is found that the urine contains conjugates of the corresponding *ortho*- or *para*-substituted phenols, or both, e.g. acetanilide (Smith & Williams, 1948), aniline (Smith & Williams, 1949), phenol (Garton & Williams, 1949) and chlorobenzene (Spencer & Williams, unpublished observations). Little definite information, however, is available about the position of biological hydroxylation of monosubstituted benzenes carrying the classical *meta*-directing groups (e.g. NO_2, CN, COOH). According to Meyer (1905) small amounts of *p*-nitrophenol occur in the urine of rabbits receiving nitrobenzene, but this was not unequivocally proved. Baumann (1883) isolated small amounts of *o*- and *p*-hydroxybenzoic acids from the acid-hydrolysed urines of dogs dosed with cyanobenzene. Evans, Parr & Evans (1949) have suggested that *p*-hydroxybenzoic acid may be the first product of the oxidation of benzoic acid by certain bacteria (see also Parr, Evans & Evans, 1949). The information available suggests that monosubstituted benzenes, if oxidized to phenols *in vivo*, give rise to *ortho*- or *para*-substituted phenols, or both, irrespective of the nature of the substituent.

Now Smith & Williams (1948) pointed out that the orientation of hydroxylation of acetanilide and aniline in the rabbit was similar to that found during nitration, i.e. *para* in acetanilide and mainly *ortho* and *para* in aniline. This suggested to us that one possible interpretation of the orientation of biological hydroxylation could be along the lines used to explain aromatic substitution in pure organic chemistry, i.e. that substitution is either ionic or free-radical in nature (for discussion of free-radical substitution see Hey & Waters, 1937, 1948; Waters, 1948; Dewar, 1949). Whilst other interpretations of the orientation of biological oxidation are also possible, we studied the fate of cyanobenzene and benzoic acid with the purely chemical theory of aromatic substitution in mind. Working on these assumptions we thought that if the orientation of hydroxylation *in vivo* were known for both *ortho*-, *para*- and *meta*-directing groups, then we could decide whether biological hydroxylation had the characteristics of ionic substitution or of free-radical substitution, assuming, of course, that the hydroxy-

lation was a substitution and not an addition reaction. The idea that free radicals may be involved in enzymic processes has been discussed by Michaelis (1946), Kalckar (1946), Delbrück (1944) and Waters (1946) among others. Regarding the question of whether it is possible to explain enzyme catalysis in terms of mesomeric free radicals, Kalckar (1946) makes the following statement: 'There are observations which may provide confirmation for such an explanation. Some years ago, Haas (1937) found that riboflavin phosphate, when linked to a protein, forms a semiquinone when undergoing reduction. This semiquinone is not observed during the reduction of free riboflavin phosphate in neutral solution, but accumulates when the reaction is acid enough to insure complete ionization. In other words, the enzyme is able to stabilize a product at neutral reaction which otherwise would exist only at strongly acid reaction. This observation may be taken as a clear indication that oxidation reduction enzymes in some way or other are concerned with the formation of mesomeric free radicals.'

EXPERIMENTAL

Preparation of reference compounds

Benzylamine salts of the cyanophenols

Equal weights of the cyanophenol and benzylamine were warmed together until a homogeneous liquid was obtained. On cooling, the mixtures containing the *o*- and *p*-isomers crystallized, whereas that containing the *m*-isomer remained liquid. The crystalline products were recrystallized from ethyl acetate, thus affording the *benzylamine salt* of *o-cyanophenol* which formed needles, m.p. 110–111°, sparingly soluble in light petroleum but soluble in water, ether, ethanol and ethyl acetate (Found: C, 73·9; H, 6·2; N, 12·3. $C_7H_5ON.C_7H_9N$ requires C, 74·3; H, 6·2; N, 12·4%); and the *benzylamine salt* of p-*cyanophenol* which formed prisms, m.p. 80°, which were very soluble in water, ether, ethanol and ethyl acetate and less soluble in light petroleum (Found: C, 73·9; H, 6·2; N, 11·9%). The *p*- was much more soluble than the *o*-derivative.

We failed to isolate a salt from *m*-cyanophenol, and the reason for this became apparent when we measured the dissociation constants of the three isomers. *m*-Cyanophenol was more feebly acidic than its isomers. The approximate pK_a's of the three cyanophenols found from their titration curves, determined with the glass electrode, were *o*-, 7·3, *m*-, 8·8 and *p*-, 8·2.

The azobenzene-4-carboxylic esters of the cyanophenols

Since it was possible that the cyanophenols of cyanobenzene urine could be separated by chromatography, it was decided to synthesize coloured derivatives of the phenols. We therefore synthesized the azobenzene-4-carboxylic esters. Equimolecular amounts of the cyanophenol and azobenzene-4-carbonyl chloride were refluxed for a few minutes with about 15 equiv. of pyridine. The solution was diluted with water and the precipitate collected and dried *in vacuo*. The solid was now extracted with $CHCl_3$ and the extract purified by passing through a short column of alumina (Peter Spence, Widnes; Grade H). The solvent was then evaporated and the residue recrystallized from a mixture of $CHCl_3$ and CCl_4.

o-Cyanophenyl azobenzene-4-carboxylate was obtained as clumps of orange needles, m.p. 153–155°, sparingly soluble in ethanol, light petroleum and CCl_4, but soluble in $CHCl_3$. (Found: N, 12·5. $C_{20}H_{12}O_2N_2$ requires N, 12·8%.) *m-Cyanophenyl azobenzene-4-carboxylate* formed sheaves of orange needles, m.p. 143–147°, of similar solubility to the 2-isomer. (Found: N, 13·1%.) *p-Cyanophenyl azobenzene-4-carboxylate* formed orange rectangular plates, m.p. 175°, with similar solubility to the other isomers. (Found: N, 13·0%.)

2:4-*Dinitrophenyl ethers of the cyanophenols*

These were prepared from cyanophenols and 1:2:4-chlorodinitrobenzene following directions given by Wild (1947).

2:4-*Dinitro-2′-cyanodiphenyl ether* formed small crossed rods, m.p. 115°. (Found: C, 55·0; H, 2·4; N, 14·9. $C_{12}H_7O_5N_3$ requires C, 54·8; H, 2·5; N, 14·7%.) 2:4-*Dinitro-3′-cyanodiphenyl ether* formed branching clusters of needles, m.p. 130°. (Found: C, 55·1; H, 2·4; N, 14·9%.) 2:4-*Dinitro-4′-cyanodiphenyl ether* formed clusters of plates, m.p. 134°. (Found: C, 55·0; H, 2·4; N, 15·2%.)

Metabolites of cyanobenzene

Preliminary experiments of a quantitative nature showed that when cyanobenzene (b.p. 188–190°/760 mm.) was fed to rabbits there was an increased output of ethereal sulphate and conjugated glucuronic acid. In two experiments it was found that 23 and 27% of the dose (145 mg./kg.) was excreted as ethereal sulphates, and 38 and 28% as conjugated glucuronic acids in 2 days. Determinations of mercapturic acid output also suggested that about 5% of the cyanobenzene was excreted as a mercapturic acid. A further metabolite appeared to be benzoic acid. It was also noted that the excretion of these metabolites persisted over 2–4 days after dosing, despite the fact that the dose was a relatively small one. It was clear, therefore, that about 60% of the cyanobenzene was hydroxylated and excreted as oxygen-conjugates.

Mercapturic acid formation

According to Baumann (1883) cyanobenzene gives rise to a mercapturic acid. Attempts were made to prove this by isolation and by quantitative estimation by the iodometric method of Stekol (1936) for dog and rat urines (see also Binkley, 1949), with slight modifications for rabbit urines. In adapting this method for various urines, attention had to be paid to the amounts of 0·5 N-NaOH and 10%(w/v) $ZnSO_4$ used for the removal of interfering substances. The correct amounts of the $ZnSO_4$-NaOH precipitants were arrived at by finding out how much of the precipitants were necessary to give the same blank titration on the filtrate from normal urines before and after alkaline hydrolysis. Daily urines (average 140 ml.) were centrifuged and made up to 200 ml. with water. 50 ml. samples were then analysed for mercapturic acid by Stekol's method using 15 ml. of 0·5 N-NaOH and 15 ml. of 10% (w/v) $ZnSO_4$ to precipitate interfering substances. Three female chinchilla rabbits, on a constant diet, were given cyanobenzene orally. The urine was analysed daily for mercapturic acid (Table 1) until the I titrations reverted to normal values. On the reasonable assumption that the I used up is due to a mercapturic acid, then rabbit

Table 1. *Mercapturic acid in the urine of rabbits receiving cyanobenzene*

(Cyanobenzene administered on the fourth day; MA = mercapturic acid.)

Rabbit no.	127	165	168
Rabbit wt. (kg.) ...	2·8	2·5	2·9
Cyanobenzene dose (mg.)	560	500	580

	Titre (0·01 N-I_2)			Titre (0·01 N-I_2)			Titre (0·01 N-I_2)		
Day	Before hydrolysis (ml.)	After hydrolysis (ml.)	MA as PhCN (mg.)	Before hydrolysis (ml.)	After hydrolysis (ml.)	MA as PhCN (mg.)	Before hydrolysis (ml.)	After hydrolysis (ml.)	MA as PhCN (mg.)
1	0·21	0·24	—	0·22	0·23	—	0·21	0·23	—
2	0·21	0·24	—	0·22	0·25	—	0·20	0·22	—
3	0·23	0·20	—	0·20	0·22	—	0·23	0·26	—
4	0·21	0·70	16·2	0·21	1·10	29·3	0·22	0·87	21·4
5	0·23	0·62	12·9	0·21	0·32	3·6	0·22	0·36	4·6
6	0·23	0·23	—	0·23	0·24	—	0·20	0·22	—

no. 127 excreted 5·3%, no. 165, 5·7% and no. 168, 5·2% of the dose of cyanobenzene as a mercapturic acid, which may be *p*-cyanophenylmercapturic acid. Attempts at isolation of the compound from rabbit urine have so far been unsuccessful and until this has been done, there is no unequivocal proof that cyanobenzene forms a mercapturic acid.

Cyanobenzene urine

In most experiments cyanobenzene, dissolved in olive oil, was fed by stomach tube. At a dose level of 150 mg./kg. no toxic effects were observed but higher doses (250 mg./kg.) were liable to be fatal. The bulk of the metabolites appeared in the urine on the second and third day after feeding. The urine reduced Benedict reagent very slightly but gave no colour with $FeCl_3$. The naphthoresorcinol test was positive. Tests for thiocyanate by $FeCl_3$ and by the $CoSO_4$ procedure of Feigl (1947) were negative. On mild hydrolysis with acid followed by ether extraction, an extract was obtained which gave a weak green colour with $FeCl_3$ chloride and reduced ammoniacal $AgNO_3$ in the cold.

This latter test suggested that a cyanocatechol may be excreted in small amounts but we were unable to isolate it and compare it with synthetic 4-cyanocatechol.

Isolation of benzoic acid

The urine (1 l.) from five rabbits which had received collectively 4 g. of cyanobenzene in aqueous suspension was collected over 2 days. After adding 100 ml. of conc. HCl, the urine was heated for 20 min. at 80°. After cooling, it was extracted with 2 × 200 ml. of ether in a separating funnel. Evaporation of the ether left an oil smelling faintly of cyanobenzene. The oil partly crystallized and by pressing on a porous tile 500 mg. of benzoic acid, m.p. and mixed m.p. 121°, were isolated.

The ethereal sulphate fraction. Isolation of m- *and* p-*cyanophenols*

(i) *Isolation of the mixed phenols.* A total of 2·8 g. of cyanobenzene in olive oil was fed to six rabbits. The urine (1·9 l.) collected over 4 days was brought to pH 4 with HCl and continuously extracted with ether for 6 hr. Evaporation of the ether left a brown tar from which nothing crystalline was isolated.

The urine was now brought to pH 1, heated at 85° for 40 min. to hydrolyse ethereal sulphates, and continuously extracted with ether for 3 hr. Evaporation of the ether left a brown tar which gave a weak green colour with $FeCl_3$ and a blue colour at pH 8 with 2:6-dichloroquinone chloroimide. Purification, but no separation, of the phenols in the tar was achieved by passing the tar dissolved in ether through a column of alumina (Peter Spence, Widnes; grade H). From the eluate 680 mg. of mixed cyanophenols (19% of dose) were obtained. Further elution of the column with methanolic HCl gave a brown tar which gave a green colour with $FeCl_3$, but we were unable to isolate cyanocatechol or protocatechuic acid from it.

The mixture of cyanophenols gave a purple colour with $FeCl_3$ and a blue colour at pH 8 with 2:6-dichloroquinone chloroimide. These tests indicated that the mixture contained an *o*- or *m*-substituted phenol, for we found that pure *o*- and *m*-cyanophenols gave a blue colour with 2:6-dichloroquinone chloroimide at pH 8, whereas the pure *p*-isomer gave no colour. 4-Cyanocatechol also gives a blue colour with this reagent. All three cyanophenols give a weak purple colour with $FeCl_3$. 4-Cyanocatechol gives the usual catechol colours with $FeCl_3$. The mixture also had a sweet taste, and it has been reported by Griess (1876) that of the three cyanophenols only the *m*-isomer has a sweet taste, a fact which we have confirmed.

(ii) *Isolation of* p-*cyanophenol.* The mixed phenols (680 mg.) were dissolved in 1 ml. of benzene and 1 ml. of light petroleum added. A crystalline precipitate of *p*-cyanophenol (125 mg. or 3·5% of dose) separated. After recrystallization from benzene it had m.p. and mixed m.p. 110–112°. (Found: N, 11·7. Calc. for C_7H_5ON: N, 11·8%.)

In another experiment 140 mg. of the mixed phenols were treated with 0·15 ml. of benzylamine and warmed until fluid. On cooling, the mixture set to a paste of crystals which, when filtered with suction and washed with light petroleum, gave 140 mg. (5% of dose and 53% of the mixed phenols) of the benzylamine salt of *p*-cyanophenol. After recrystallization from a small amount of ethyl acetate, the salt had m.p. and mixed m.p. 80° with an authentic specimen (cf. p. 244). (Found: C, 74·8; H, 6·3; N, 11·8%.)

(iii) *Isolation of* m-*cyanophenol.* The mixed phenols (600 mg.) were warmed with benzylamine (500 mg.) until liquid and the product was cooled to −15° for 0·5 hr. Crystals of the benzylamine salt of *p*-cyanophenol (420 mg. or 37% of the mixture) were washed with a mixture of equal volumes of light petroleum and benzene and dried with suction. The filtrate was evaporated to dryness and the residue dissolved in 10 ml. of ether and extracted with 2 × 5 ml. of 2 N-HCl to

remove benzylamine. The ether was extracted with 8×2 ml. of 2N-Na_2CO_3, and the alkaline extracts were acidified with HCl and extracted with ether. After drying, removal of the solvent left a crystalline residue (140 mg., m.p. 50–55°) which gave a weak purple colour with $FeCl_3$ and a strong blue colour with 2:6-dichloroquinone chloroimide in alkaline solution. The product (120 mg.) was then refluxed for 1 hr. with 0·5 ml. of 2N-NaOH and 200 mg. of 1:2:4-chlorodinitrobenzene, and the pasty precipitate (65 mg.) was washed with ether and filtered. On recrystallization twice from ethanol the characteristic branched needles of 2:4-dinitro-3′-cyanodiphenyl ether were obtained. The m.p. and mixed m.p. was 130–131°. The m.p. was considerably depressed by the corresponding *o*- and *p*-cyanophenol derivatives. (Found: C, 54·95; H, 2·1; N, 14·4%.) The amount of *m*-cyanophenol isolated here corresponded to 5% of the mixed phenols.

The presence of *m*-cyanophenol was confirmed by the isolation of its azobenzene-4-carbonyl ester. The ethereal solution left after extraction with 2N-Na_2CO_3 (see above) was evaporated to dryness. The residue (100 mg.) was boiled for a few minutes with 200 mg. of azobenzene-4-carbonyl chloride in 2 ml. of pyridine. Dilution to 50 ml. with water yielded a precipitate which was collected and dried *in vacuo*. The dried material was now purified by dissolving in $CHCl_3$, filtering and passing through a short column of alumina (Peter Spence, Widnes, grade H). Evaporation of the eluate after washing the column with $CHCl_3$ left an orange residue (50 mg. or 3% of the mixed phenols) which crystallized from $CHCl_3$ as orange plates (26 mg.), m.p. 145–146° not depressed by authentic *m*-cyanophenyl azobenzene-4-carboxylate but considerably depressed by the corresponding esters of *o*- and *p*-cyanophenol. (Found: C, 73·0; H, 4·1; N, 12·7%.)

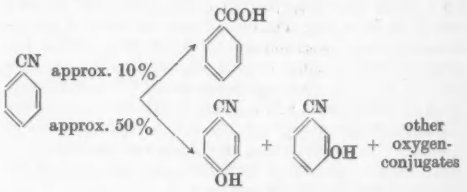
Search for oxidation products of benzoic acid

The main metabolites of benzoic acid are known to be hippuric acid and benzoylglucuronide, and since it does not cause increased excretion of ethereal sulphates in the rabbit (Bray, Neale & Thorpe, 1946) it seems unlikely that it is oxidized to phenolic acids. However, the possible oxidation products are *o*-, *m*- and *p*-hydroxybenzoic acids and protocatechuic acid. Now salicylic acid is known not to form an ethereal sulphate in the rabbit (Williams, 1938; Bray, Ryman & Thorpe, 1948), but it is easily detectable if present. The *m*- and *p*-hydroxybenzoic acids have low sulphate conjugations in the rabbit (Williams, 1938; Hartles & Williams, 1948; Bray *et al.* 1948). Should any of these acids be formed metabolically from benzoic acid, their formation could not be detected by ethereal sulphate determinations. Protocatechuic acid, however, does conjugate appreciably with sulphate in the rabbit (Dodgson, Garton & Williams, 1947; Dodgson & Williams, 1949). Benzoic acid was therefore fed to rabbits and the urine fractionated. The fractions were examined in detail for salicylic acid and protocatechuic acid by colour reactions and for *m*- and *p*-hydroxybenzoic acids spectroscopically.

Although the urine was examined in considerable detail, the only compounds found were hippuric acid, benzoylglucuronide and free benzoic acid. We found no hydroxybenzoic acids.

DISCUSSION

In the present paper we have not dealt with the glucuronic acid fraction of cyanobenzene urine. Although we have examined this fraction several times we have been unable to elucidate its nature except to show that a small part of it is benzoylglucuronide. However, as far as we have gone, the metabolites of cyanobenzene in the rabbit are *m*- and *p*-cyanophenols, which are excreted as ethereal sulphates, and benzoic acid, which is partly combined with glycine and glucuronic acid. Some of our evidence also suggests the presence of traces of a catechol derivative and small amounts of a mercapturic acid. We were unable to detect thiocyanates which are metabolites of aliphatic cyanides and derivatives of benzyl cyanide (Williams, 1947). Of the earlier work on cyanobenzene, Giacosa (1883) showed that it formed small amounts of benzoic acid in the dog; Baumann (1883) hydrolysed cyanobenzene urine from dogs and isolated small amounts of *o*- and *p*-hydroxybenzoic acids; and Adeline, Cerecedo & Sherwin (1926) failed to detect any metabolites in rabbits. Accordingly, the fate of cyanobenzene in the body may follow three paths: (*a*) oxidation to phenols, (*b*) hydrolysis to benzoic acid, and (*c*) possibly mercapturic acid formation. The third path is suggested tentatively and requires further confirmation. On the present evidence the following scheme may be suggested:



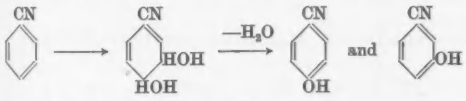
Although the work of Baumann (1883) suggests that cyanobenzene may undergo oxidation in the *ortho* position, we have not been able to detect *o*-cyanophenol or salicylic acid in our experiments.

There is a sharp distinction between benzoic acid and cyanobenzene in that it appears that the latter alone suffers oxidation in the body, a difference which obviously requires some explanation. Lederer (1949) (Lederer & Polonsky, 1948) has recently reported that *o*-, *m*- and *p*-hydroxybenzoic acids are constituents of normal urine. All three acids have been isolated from the urine of pregnant mares and the scent gland of the beaver. Furthermore, Lederer refers to the observation of Bielig & Hayasida (1940), who isolated *m*-hydroxybenzoic acid from rabbit urine. These acids could have arisen by the hydroxylation *in vivo* of benzoic acid, although other explanations are not excluded, and our negative results may have been due to not working on a large enough scale. However, it is clear that if benzoic acid is oxidized *in vivo*, then the extent of oxidation must be a very small one. Now it appears that benzoic acid is rapidly excreted, whereas cyanobenzene is

very slowly eliminated. There may therefore be a time factor involved, and consequently cyanobenzene has more opportunity of being oxidized than benzoic acid. Furthermore, the elimination of benzoic acid is assisted by its rapid conjugation with glycine and glucuronic acid to form conjugates which are quickly eliminated from the body. It is also possible that benzoic acid does not reach the site of hydroxylation in the body if this is different from that of conjugation, for relatively strongly ionized compounds do not penetrate cell membranes (Höber, 1946). Evidence in support of the suggestion that a time factor is involved can be gathered from the facts that salicylic acid, a substance which is slowly eliminated from the body (Quick, 1933; Kapp & Coburn, 1942) is in fact hydroxylated to gentisic acid (Baldoni, 1908; Angelico, 1921; Kapp & Coburn, 1942; Bray *et al.* 1948), and that benzoic acid is hydroxylated by certain soil bacteria when it is added to the medium in which the organisms are growing and is thus in contact with the organisms for a prolonged period (Evans, 1947).

The important observations made in this work are, first, that *meta*-hydroxylation appears to occur in the body, and secondly, that although cyanobenzene carries a *meta*-directing group it appears to undergo *para*-hydroxylation as do those monosubstituted benzenes carrying *ortho-para*-directing groups. Further work (unpublished) in this laboratory indicates that nitrobenzene is also *meta*-hydroxylated for we have isolated *m*-nitrophenol from the urine of rabbits receiving nitrobenzene and found strong indications that the *para*-isomer is also formed. The rest of this discussion will deal with how these observations could be interpreted in the light of current theories of diol formation *in vivo* (cf. Boyland, 1949; Boyland & Weigert, 1947) and of aromatic substitution.

In the first place it is possible that the *m*- and *p*-cyanophenols could have arisen during the isolation procedures as the result of dehydration by acid of a cyanodihydrobenzene-3:4-diol thus:



Dihydrodiols of polycyclic hydrocarbons are known to undergo this reaction (Young, 1947; Boyland & Wolf, 1948) although only one phenol, and not two as above, is produced in the cases of the diols of naphthalene and anthracene (for the action of dilute acid on the diols of other polycyclic hydrocarbons, see Cook & Schoental, 1948). However, we looked for a diol in cyanobenzene urine but did not detect it. It seems likely that the *m*- and *p*-cyanophenols were present in the urine mainly as ethereal sulphates because about 25% of the cyanobenzene fed was excreted as ethereal sulphate. It is possible that they were produced metabolically from a diol (cf. Berenblum & Schoental, 1949).

If our results are considered from the standpoint of the rules of aromatic substitution, the predicted orientation for anionic substitution of cyanobenzene would be *ortho* and *para*, whereas in the case of aniline, for example, the orientation would be *meta*. In the case of cationic substitution the predicted orientation would be *meta* for cyanobenzene and *ortho* and *para* for aniline. Now it has been found that, in the rabbit, cyanobenzene is *meta*- and *para*-hydroxylated, whereas aniline is *ortho*- and *para*-hydroxylated (Smith & Williams, 1949). These orientations cannot be explained in terms of either anionic or cationic substitution alone, although they can, if both mechanisms were assumed to act simultaneously, which, however, is very unlikely. However, it is known, in purely chemical reactions, that abnormal orientations occur when the reagent used gives rise to free radicals and in these cases all three positions may be substituted, the *para* being the more favoured, irrespective of the normal directing properties of the existing substituent for ionic substitutions. It appears, therefore, that the biological orientations found with cyanobenzene and aniline are analogous with those found during free-radical substitution. The question now arises whether the occurrence of abnormal substitution can be considered as evidence for the participation of neutral free radicals. It appears to us that there is a tendency to accept this for certain purely chemical reactions (cf. Hey, 1941; Dewar, 1949; Loebl, Stein & Weiss, 1949). Whether this is also true for biochemical reactions may be disputed. In this connexion it is interesting to note that Stein & Weiss (1948) have suggested that hydroxyl radicals may play an important part in certain biological oxidations. They found that when solutions of benzene and benzoic acid were submitted to the action of hydroxyl radicals produced by radiations, these compounds were hydroxylated and the products were similar to those obtained in the biological oxidation of these substances.

SUMMARY

1. A study has been made of the hydroxylation of cyanobenzene and benzoic acid in the rabbit.

2. No evidence was obtained of the oxidation of benzoic acid; the only metabolites obtained here were the unchanged acid, its glycine derivative and glucuronide.

3. Cyanobenzene is very slowly eliminated and undergoes considerable change *in vivo*. About 60% is excreted as oxygen conjugates, 10% is transformed to benzoic acid and it is suggested that another 5% forms a mercapturic acid.

4. The ethereal sulphate fraction of cyanobenzene urine contains *m*- and *p*-cyanophenols, which were isolated. The formation of *m*-cyanophenol is believed to be the first reported case of *meta*-hydroxylation *in vivo*. The nature of the glucuronide fraction was not elucidated.

5. The azobenzene-4-carbonyl esters, 2:4-dinitrophenyl ethers and benzylamine salts of the cyanophenols have been synthesized and used as reference compounds. The pK_a's of *o*-, *m*- and *p*-cyanophenols have been measured.

6. The results have been discussed in the light of current theories concerning diol formation *in vivo* and aromatic substitution *in vitro*.

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The Oxidation of Pyruvate and Fatty Acids by *Mycobacterium ranae*

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A study of the intermediary metabolism of *Mycobacterium phlei* led Edson & Hunter (1947) to suggest that the aerobic breakdown of lactate may proceed through pyruvate to acetate by two routes, one of which involves a coupled non-enzymic oxidation of pyruvate. The alternative pathway via enzymic oxidation of pyruvate requires investigation, because it offers the possibility of partial assimilation at an early stage. Since added pyruvate is metabolized slowly by washed suspensions of *Mycobact. phlei*, we decided to study the oxidation of this substrate by *Mycobact. ranae* which utilizes both pyruvate and

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acetate at more favourable rates (Edson & Hunter, 1943). Some related observations on the oxidation of fatty acids have been included in this paper.

METHODS

Organism. The organism used was *Mycobact. ranae* no. 2891 of the National Collection of Type Cultures, Lister Institute, London. It was cultivated at 38° on meat infusion broth, pH 7·2, containing 5% (v/v) of glycerol. On the seventh to tenth day of incubation the bacteria were harvested, washed and suspended in distilled water according to Edson & Hunter (1943).

Bacterial extracts. Extracts and solid cellular debris were prepared with the wet-crushing mill of Booth & Green (1938) or by the glass-powder grinding technique of Kalnitsky, Utter & Werkman (1945), and separated by centrifugation.

Measurement of respiration. Oxygen consumption and CO_2 production were measured at 38° by the conventional manometric methods of Warburg. Substrates (as neutral Na salts) were added from the side bulb after equilibration. The quantity of bacteria used varied from 6 to 20 mg. (dry wt.)/cup.

Analytical methods

(*a*) *Determination of pyruvate.* After the Warburg cups had been cooled in ice and the CO_2 absorbent removed, the contents were transferred to graduated centrifuge tubes, the vessels rinsed with 1 ml. of distilled water and the washings added to the centrifuge tubes. The volume was measured and the bacteria thrown down by centrifugation at 4500 rev./min.; 3·0 ml. of supernatant fluid were used for determination of pyruvic acid by the yeast carboxylase method of Westerkamp (1933), and the result corrected to give the full value for each Warburg cup.

(*b*) *Determination of fatty acids volatile in steam.* At the conclusion of a manometric experiment the Warburg cups were cooled in ice, the CO_2 absorbent removed and the bacterial suspension transferred to a graduated test tube by means of a pasteur pipette. The main compartment, the side bulb and the pipette were washed with small amounts of distilled water (3 ml. in all), and the washings added to the test tube; 0·5 ml. of 10% (w/v) Na tungstate solution and 0·5 ml. of 0·66 N-H_2SO_4 were added and the volume made up to 8·0 ml. with distilled water. The mixture was filtered through a Whatman no. 1 filter paper (4·25 cm. diameter) and 7·0 ml. of the clear filtrate were used for steam distillation and estimation of fatty acids by the methods of Friedemann (1938). When identification was required, Duclaux fractions were titrated and distribution constants (2 vol. distilled water/1 vol. ether) determined. The apparatus blank was 0·25 ml. of 0·01 N-NaOH.

Table 1 gives typical values obtained with the particular apparatus and specimens of fatty acids employed. The acetate was A.R. (British Drug Houses Ltd.), $CH_3COONa3H_2O$. The serial distillate figures agree satisfactorily with those of Friedemann (1938), except in the case of acetic acid where the percentages in the fifth and sixth fractions are significantly different. The distribution constants (K) are uniformly higher than those given by Friedemann (1938).

Table 1. *Steam distillation of fatty acids. Duclaux fractions and distribution constants*

(Method of Friedemann (1938). Data obtained with the apparatus and specimens of fatty acid used in subsequent work.)

Fraction no.	1	2	3	4	5	6	
Volume of fraction (ml.)...	25	25	25	25	50	About 40*	Total distillate
	Percentage of fatty acid						Distribution constant K (2 vol. water/1 vol. ether)
Acetic acid	12	12	14	14	37	11	90
Propionic acid	26	23	19	16	14	2	71
n-Butyric acid	38	27	16	10	6	3	46
n-Valeric acid	—	—	—	—	—	—	32
n-Hexanoic acid	78	18	3	1	Nil	Nil	11

* To crystallization.

RESULTS

Respiration of Mycobacterium ranae

Table 2 shows that *Mycobact. ranae* possesses a substantial endogenous respiration and that oxygen consumption is increased about four times after

Table 2. *Respiration of* Mycobacterium ranae *in presence of pyruvate and fatty acids*

(Washed bacterial suspension in 3·0 ml. of 0·033 M-phosphate buffer pH 7·4. 38°. Gas: air. $-Q_{O_2}$ = µl. O_2 consumed/mg. dry wt. of bacteria/hr.)

Substrate (0·02 M)	$-Q_{O_2}$ Substrate absent	$-Q_{O_2}$ Substrate present
Pyruvate	8·1	34·6
Acetate	8·1	36·8
Propionate	8·5	74·5
n-Butyrate	8·8	79·0
n-Valerate	5·8	41·0
Pentane-1-carboxylate	8·4	70·0

Mean values (with S.D.) of twenty observations were as follows:

Substrate	$-Q_{O_2}$
Nil	8·7 ± 2·4
Pyruvate	34·1 ± 4·2
Acetate	35·6 ± 4·9

Table 3. *Incomplete oxidation of pyruvate and fatty acids by* Mycobacterium ranae

(Washed bacterial suspension in 3·0 ml. of 0·033 M-phosphate buffer pH 7·4. 38°. Gas: air.)

Substrate (M)	Dry wt. bacteria/cup (mg.)	Time (hr.)	O_2 uptake (μl.)	'Extra' O_2 uptake (μl.)	Substrate disappearing (mg. free acid)	Mol. 'extra' O_2 used/mol. substrate disappearing: Found	Mol. 'extra' O_2 used/mol. substrate disappearing: Theoretical for complete combustion
Nil	10·5	5	302	—	—	—	—
Pyruvate (0·02)			1606	1304	3·31	1·5	2·5
Nil	7·25	4	155	—	—	—	—
Acetate (0·02)			1072	917	1·65	1·5	2·0
Nil	6·9	4	230	—	—	—	—
Propionate (0·025)			2089	1859	2·94	2·1	3·5
Nil	7·1	4	225	—	—	—	—
n-Butyrate (0·02)			1369	1144	2·03	2·2	5·0
Nil	7·5	4	175	—	—	—	—
n-Valerate (0·017)			1235	1060	1·17	4·1	6·5
Nil	8·2	3	291	—	—	—	—
Pentane-1-carboxylate (0·016)			2433	2142	2·19	5·1	8·0

addition of pyruvate or acetate, these substrates being oxidized at approximately equal rates. In the presence of propionate, butyrate, valerate and pentane-1-carboxylate the oxygen uptake is increased eight- to nine-fold. Bacterial suspensions which had been heated for 3 min. at 100° exhibited no oxygen uptake either in presence or absence of substrate.

Incomplete oxidation of substrates

The incompleteness of combusion of pyruvate and fatty acids is illustrated by the experiments recorded in Table 3. The ratio oxygen consumed/substrate disappearing has been calculated on the assumption that the endogenous respiration is unaffected by the metabolism of the substrate. The figures show that the oxygen uptake/mol. of substrate utilized is considerably less than that required for complete combustion. A similar incomplete oxidation of glucose and lactate was observed with *Mycobact. phlei* (Edson & Hunter, 1947). Since the relationship between pyruvate and acetate is an important feature of the metabolism of acid-fast bacteria, acetate was sought, but not found, in the suspension medium during the metabolism of pyruvate. With the exception of CO_2, no product of pyruvate catabolism is known to accumulate extracellularly. It is to be noted, however, that the combustion of acetate is more complete than that of pyruvate (75% as against 60%). The data of Table 3 suggest that a portion of each substrate is burnt to completion and the remainder assimilated.

Anaerobic dissimilation

When pyruvate and acetate were added anaerobically to a suspension of *Mycobact. ranae*, the CO_2 production was not significantly different from the small evolution occurring in the control without substrate (Table 4). There was a slight increase in CO_2 formation in the presence of glucose. Determination of residual pyruvate and acetate in experiments lasting 4 hr. showed that there was no significant metabolism of these substrates under anaerobic conditions. The recoveries were 95% or more of the amounts added to the bacteria.

Table 4. *Anaerobic evolution of carbon dioxide by* Mycobacterium ranae

(Washed bacterial suspension in 3·0 ml. of bicarbonate-Ringer solution (Krebs & Henseleit, 1932); pH 7·4; 38°. Gas: 5% CO_2–95% N_2. Phosphorus in the inseal. Dry wt. of bacteria = 7 mg./cup.)

Substrate (0·02 M)	CO_2 production (μl./hr.)	Recovery of substrate (%)
Nil	21	—
Pyruvate	21	95
Acetate	29	99
Glucose	41	—

Inhibition of substrate oxidation

The effects of various reagents on the oxidation of pyruvate, acetate, butyrate and pentane-1-carboxylate are shown in Tables 5 and 6. The percentage inhibition of respiration is calculated for the oxygen uptake in excess of that due to endogenous respiration. The effects of inhibitors on the spontaneous respiration are also recorded. It will be seen that the oxidation of pyruvate and acetate is partially inhibited by azide at the concentration used; cyanide has a more pronounced effect, and 0·005 M-cyanide gives almost complete inhibition in the presence of acetate. Fluoride diminishes the extra oxygen consumption by 32% in the presence of pyruvate, but has no effect when acetate is the substrate. The results with malonate are similar.

Fluoroacetate assumed importance as a selective inhibitor in studies of intermediary metabolism when Barron, Bartlett & Kalnitsky (1946), Bartlett & Barron (1947) and Kalnitsky & Barron (1947) reported that this substance inhibited acetate oxidation without inhibiting the oxidation of pyruvate in animal tissues, in baker's yeast and in *Corynebacterium creatinovorans*. Since fluoroacetate depresses the oxygen consumption of *Mycobact. ranae* to the same degree (approximately 30% in a 2 hr.

Table 5. *Inhibition of the oxidation of fatty acids by* Mycobacterium ranae

(Washed bacterial suspension in 3·0 ml. of 0·033 M-phosphate buffer pH 7·4. 38°. Gas: air. Inhibitor in contact with the bacteria from the beginning of the experiment; substrate added after equilibration. When HCN was used, the CO_2 absorbent was a mixture of KCN and KOH (Krebs, 1935).)

Inhibitor	Dry wt. of bacteria/cup (mg.)	Time (hr.)	Oxygen uptake (μl.) Substrate absent	Oxygen uptake (μl.) Substrate present Acetate (0·02 M)	Inhibition (%)
Nil	10·4	2	175	591	—
Sodium azide (0·002 M)			179	445	36
HCN (0·005 M)			105	121	96
Sodium fluoroacetate (0·005 M)			178	438	38
Nil	7·2	2	118	533	—
Sodium fluoride (0·005 M)			120	542	−2
Sodium malonate (0·01 M)			126	583	−9
Nil	14·0	4	454	2061	—
Hydroxylamine-HCl (neutralized) (0·02 M)			415	574	90
Nil	7·5	2	151	593	—
Sodium arsenite (0·004 M)			126	127	100
Nil	6·7	2	142	596	—
Sodium iodoacetate (0·002 M)			130	152	95
Sodium iodoacetate (0·001 M)			144	155	98
				n-Butyrate (0·02 M)	
Nil	6·7	2	142	758	—
Sodium iodoacetate (0·002 M)			130	156	96
				Pentane-1-carboxylate (0·016 M)	
Nil	8·4	2	128	1110	—
Sodium arsenite (0·004 M)			95	189	90
Nil	10·0	7	407	2839	—
Sodium iodoacetate (0·002 M)			408	759	86

Table 6. *Inhibition of the oxidation of pyruvate by* Mycobacterium ranae

(Washed bacterial suspension in 3·0 ml. of 0·033 M-phosphate buffer pH 7·4. 38°. Gas: air. Inhibitor in contact with the bacteria from the commencement of the experiment; substrate added after equilibration. Final conc. pyruvate = 0·02 M.)

Inhibitor	Time (hr.)	Oxygen uptake (μl.) Substrate absent	Oxygen uptake (μl.) Substrate present	Inhibition (%)
Nil	2	106	425	—
Sodium azide (0·002 M)		105	341	26
HCN (0·001 M)		80	212	59
Nil	1	80	319	—
Sodium fluoride (0·005 M)		82	245	32
Nil	1	88	470	—
Sodium malonate (0·01 M)		91	393	21
Nil	2	201	837	—
Sodium fluoroacetate (0·005 M)		221	671	29
Nil	4	454	1939	—
Hydroxylamine-HCl (neutralized; 0·01 M)		415	2022	−8
Nil	1	76	303	—
Sodium arsenite (0·005 M)		64	85	91
Nil	1	78	317	—
Sodium iodoacetate (0·002 M)		69	159	63

experiment) whether the substrate undergoing oxidation be acetate or pyruvate, it is unlikely to be a useful agent in attempts to dissociate their metabolism. Moreover, the inhibitory action decreases with time. Inhibitions of the order of 50% have been observed in the first hour, only to diminish to negligible effects after 3 or 4 hr. Progressive diminution of the specific inhibitory action of fluoroacetate has been reported by Kalnitsky & Barron (1947) and by Black & Hutchens (1948) in studies of baker's yeast.

As with *Mycobact. phlei* (Edson & Hunter, 1947), arsenite inhibits the oxidation of both pyruvate and acetate completely or almost completely. It has the same effect on the oxidation of pentane-1-carboxylate (Table 5).

Two of the reagents investigated exhibit selective properties which are potentially useful. Hydroxylamine, which does not affect oxygen uptake significantly in the presence of pyruvate, causes almost full inhibition of acetate oxidation. Equally noticeable is the influence of iodoacetate, which reduces extra oxygen uptake by 95–100% in the presence of acetate, but only by 63% in the presence of pyruvate. Iodoacetate has a similar profound effect on the oxidation of other fatty acids, 96% inhibition in the case of butyrate and 86% in that of pentane-1-carboxylate.

Dissociation of the metabolism of pyruvate and acetate. The time course of the respiration of *Mycobact. ranae* in presence of pyruvate and acetate is illustrated in Fig. 1, which represents an experiment lasting 7 hr. The endogenous respiration diminished slowly from the commencement, whereas oxygen consumption was maintained for 3 hr. at a constant rate in the presence of both substrates, but fell off subsequently. Iodoacetate produced a slight depression of the endogenous respiration. Addition of acetate did not affect the oxygen consumption of cells poisoned with iodoacetate; whereas the addition of pyruvate was followed by an extra oxygen uptake amounting to 16% of that which occurred in control organisms without iodoacetate. This experiment shows that the selective action of iodoacetate on the metabolism of *Mycobact. ranae* is such that it can be employed to dissociate the oxidation of acetate from that of pyruvate. If the reaction, pyruvate → acetate + CO_2, is the sole, or the chief, reaction persisting in cells poisoned with iodoacetate, the residual metabolism should be capable of producing measurable quantities of acetic acid in experiments of 5–7 hr. duration.

When a small initial concentration (0·005M) of pyruvate was used in an experiment without inhibitor, the extra oxygen uptake was rapid during the first hour, but declined to the endogenous level in 180 min. By this time the whole of the pyruvate had disappeared from the suspension medium (nitroprusside reaction negative), showing that the organism is able to utilize pyruvate completely although this substrate is not completely burnt (Table 3).

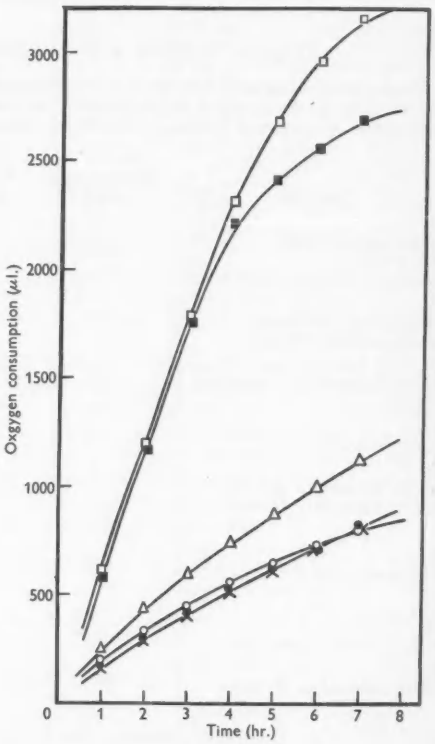

Fig. 1. Effect of iodoacetate on the oxygen consumption of *Mycobact. ranae* in presence of pyruvate and acetate. Washed suspension of organisms in 3·0 ml. of 0·033M-phosphate buffer pH 7·4. 38°. Gas: air. 21·0 mg. dry wt. of bacteria/Warburg cup. Pyruvate and acetate, each 0·02M. Iodoacetate, 0·002M. No substrate, ○; iodoacetate, ●; acetate, □; pyruvate, ■; acetate + iodoacetate, ×; pyruvate + iodoacetate, △. The curves representing the effects of iodoacetate alone and of iodoacetate + acetate are almost identical.

Formation of acetate from pyruvate. If *Mycobact. ranae* oxidizes pyruvate to acetate, the reaction is likely to proceed according to the over-all equation

$$CH_3.CO.COOH + \tfrac{1}{2}O_2 = CH_3.COOH + CO_2.$$

The results of a typical experiment designed to test this hypothesis are set out in Table 7, iodoacetate being employed to inhibit acetate oxidation selectively. In the absence of inhibitor pyruvate and acetate are oxidized vigorously, and the disappear-

ance of acetate reaches the expected order of magnitude. Acetate does not accumulate as a result of endogenous respiration or of pyruvate metabolism. The inhibitor, however, depresses oxygen consumption in the presence of pyruvate, and simultaneously causes the accumulation of significant amounts of steam-volatile acid. The oxygen uptake and the recovery figures for acetic acid show that the metabolism of acetate is abolished. It will be seen that a small quantity of steam-volatile acid accumulates in a suspension to which inhibitor alone has been added (vessel 6).

The steam-volatile acid formed from pyruvate and accumulating in vessel 3 was subjected to analysis by the methods of Friedemann (1938). The Duclaux constants and the distribution constant (Table 7) indicate that it was a specimen of acetic acid which did not contain significant amounts of other steam-volatile acids (cf. Table 1).

Controls. The possibility of errors arising in the steam distillations was controlled by investigation of the reagents, separately and combined, and by conducting blanks with suspensions of the organisms, both viable and heated (100°). Neither pyruvate nor iodoacetate yields acid volatile in steam from solutions put through the whole procedure used in this work, even when the concentration is several times greater than that used in experiments. Suspensions of *Mycobact. ranae* shaken at 38° for 7 hr. and subsequently clarified by tungstic acid precipitation, yield a trace of steam-volatile acid equal in titre to 0·1–0·2 ml. of 0·01 N-NaOH. This blank, which is constant over a density range of 6–25 mg. of bacteria (dry wt.) per 4 ml. of medium, was deducted in titrations of steam-volatile acid.

When these controls are taken into account, it appears justifiable to conclude that the trace of steam-volatile acid found in vessel 6 (Table 7) was a true metabolic product formed endogenously under the influence of the inhibitor. There was insufficient of this acid to permit identification by fractional distillation, but the lanthanum-iodine colour test (Table 8) indicates that it was probably acetic acid.

Table 7. *Formation of acetate from pyruvate in the presence of iodoacetate by* Mycobacterium ranae

(Washed bacterial suspension in 3·0 ml. of 0·033 M-phosphate buffer pH 7·4. 38°. Gas: air. Time: 7 hr. Dry wt. of bacteria/cup 21·0 mg.)

Vessel no.	Substrate (0·02 M)	Iodoacetate (0·002 M)	O_2 uptake (μl.)	Acetic acid added (mg.)	Volatile acid found reckoned as CH_3COOH (mg.)
1	Nil	Absent	800	Nil	0·00
2	Pyruvate	Absent	2701	Nil	0·00
3	Pyruvate	Present	1122	Nil	1·45
4	Acetate	Absent	3166	4·60	0·72
5	Acetate	Present	814	4·60	4·70
6	Nil	Present	816	Nil	0·20

Constants of the volatile acid formed from pyruvate in vessel 3

Duclaux fractions: Fraction no.	Duclaux fractions: Percentage of total fatty acid	Distribution constant K (2 vol. water/1 vol. ether)
1	14	90
2	12	
3	12	
4	13	
5	38·5	93
6	10·5	

Table 8. *Formation of acetate from pyruvate by* Mycobacterium ranae

(4·9 g. of washed bacteria suspended in 30 ml. distilled water and 10 ml. of the suspension heated at 100° for 5 min. Experiment conducted in 50 ml. stoppered conical flasks shaken in a thermostat at 38°. Time: 6 hr. Gas: air. The contents of each vessel were deproteinized and cleared of cells by addition of 1·0 ml. of 7% (w/v) metaphosphoric acid followed by filtration. Steam distillates were used for the La-iodine reaction (see Edson & Hunter, 1947).)

Flask no.	1	2	3	4	5	6
	Volume (ml.)					
Bacterial suspension	4·0	4·0	4·0	4·0	0	0
Heated bacterial suspension	0	0	0	0	4·0	4·0
Phosphate buffer (pH 7·4; 0·1 M)	1·5	1·5	1·5	1·5	1·5	1·5
Pyruvate (0·2 M)	0	0·8	0	0·8	0	0·8
Sodium iodoacetate (0·05 M)	0	0	0·7	0·7	0·7	0·7
Distilled water	1·5	0·7	0·8	0	0·8	0
Total volume	7·0	7·0	7·0	7·0	7·0	7·0
Lanthanum-iodine test	–	–	Trace	++++	—	—

Experiments similar to those reported in Table 7 were conducted on a larger scale for the purpose of applying the lanthanum-iodine colour test for the identification of acetic acid (see Table 8). The steam distillates from suspensions without substrate and from those to which pyruvate alone was added gave no colour reaction, whereas an intense blue coloration was obtained from distillates containing the products of metabolism of pyruvate in the presence of iodoacetate. The colour obtained from distillates representing endogenous metabolism under the influence of iodoacetate was a faint purplish blue. Heat-inactivated organisms used as controls gave negative reactions. Controls with pyruvate and iodoacetate solutions were also negative.

In view of the data which are presented in Tables 7 and 8 it may be concluded that the steam-volatile acid formed during the oxidation of pyruvate is indeed acetic, and not propionic, acid.

Molecular relationships in the metabolism of pyruvate

By measuring simultaneously pyruvate disappearance, oxygen consumption and acetate formation, it is possible to obtain some further insight into the metabolism of pyruvate. Warburg vessels were set up in duplicate for determination of pyruvic and acetic acids, the density of suspended organisms and other conditions being uniform. The results are displayed in Table 9. In absence of iodoacetate the disappearance of 37·6 μmol. of pyruvate corresponds to an extra oxygen uptake of 58·2 μmol., giving a ratio O_2 uptake/pyruvate used = 1·55. When iodoacetate is present the pyruvate disappearance is reduced to 9·5 μmol. and the extra oxygen consumption to 6·0 μmol. Under these conditions the ratio, O_2 uptake/pyruvate used = 0·63. It will be noticed that in the presence of iodoacetate the steam-volatile acid formed endogenously, assumed not without reason to be acetic acid, is almost half that produced when pyruvate is present.

Again these figures illustrate that the unhindered oxidation of pyruvate is incomplete, the theoretical oxygen/pyruvate ratio for complete combustion being 2·5. When acetate oxidation is suppressed by the selective inhibitor, the oxygen/pyruvate ratio is slightly greater than the theoretical 0·5, although the deviation is close to the limit of experimental error. If the inhibition of acetate oxidation is not quite complete, the ratio will move in the direction found. That pyruvate disappearance (9·5 μmol.) is not equal to accumulation of acetate (8·3 μmol.) is also consistent with such an explanation.

Table 9. *Relationship between oxygen consumption, pyruvate disappearance and acetate formation by* Mycobacterium ranae

(Washed bacterial suspension in 3·0 ml. of 0·033 M-phosphate buffer pH 7·4. 38°. Gas: air. Time: 5 hr. Dry wt. of bacteria/cup 10·5 mg.)

Substrate	Iodoacetate (0·002 M)	O_2 uptake (μmol.)	'Extra' O_2 consumed (μmol.)	Pyruvate added (μmol.)	Pyruvate disappearing (μmol.)	Acetic acid formed (μmol.)	'Extra' acetic acid (μmol.)	$\frac{\mu\text{mol. 'Extra' } O_2}{\mu\text{mol. Pyruvate disappearing}}$
Nil	Absent	13·5	—	—	—	0·00	—	—
Nil	Present	13·2	—	—	—	8·0	—	—
Pyruvate	Absent	71·7	58·2	47·3	37·6	0·00	—	1·55
Pyruvate	Present	19·2	6·0	47·3	9·5	16·3	8·3	0·63
Theoretical molar relationships:								
Complete combustion of pyruvate		—	2·5	1·0	1·0	—	0·0	2·5
Conversion of pyruvate to acetate		—	0·5	1·0	1·0	—	1·0	0·5

Table 10. *Oxygen consumption and carbon dioxide production in presence of iodoacetate by* Mycobacterium ranae

(Washed bacterial suspension in 3·0 ml. of 0·033 M-phosphate buffer. pH 7·4. 38°. Gas: air. O_2 uptake and CO_2 production (free and bound) measured by the method of Warburg & Yabusoe (1924).)

Substrate (0·02 M)	Dry wt. of bacteria/cup (mg.)	Time (min.)	Iodoacetate (0·002 M)	O_2 uptake (μl.)	CO_2 production (free and bound) (μl.)	CO_2/O_2	$\frac{\text{'Extra' } CO_2}{\text{'Extra' } O_2}$	Theoretical R.Q.
Nil	7·0	80	Absent	91	72	0·79	—	—
Acetate			Absent	220	215	0·98	1·11	1·0
Nil	8·2	60	Absent	78	56	0·72	—	—
Pyruvate			Absent	275	361	1·31	1·55	1·2
Nil	10·0	120	Present	147	115	0·78	—	—
Pyruvate			Present	220	266	1·21	2·06	2·0*

* It is assumed that pyruvate is converted into acetate quantitatively in the presence of the inhibitor (iodoacetate).

The data of Table 9 show that pyruvate is oxidized mainly, or solely, to acetate (and CO_2) when the metabolism is deranged by iodoacetate. They also reveal another fact of considerable importance: the utilization of pyruvate is four times as great in the absence of iodoacetate as in its presence. Since the inhibitor practically abolishes the metabolism of acetate (added or formed from pyruvate), this suggests that the normal assimilation of pyruvate may be coupled with the oxidation of acetate, or that there may be an independent oxidative assimilation of pyruvate which is highly sensitive to iodoacetate, and possibly to other inhibitors like arsenite and cyanide.

Additional experiments relevant to the molecular relationships are reported in Table 10. When oxygen consumption and CO_2 production were measured simultaneously in the presence of pyruvate and iodoacetate, the observed CO_2/O_2 ratio (2·06) was consistent with complete blockage of assimilation and quantitative conversion of the substrate into acetate. Control experiments with uninhibited organisms gave CO_2/O_2 ratios which are somewhat higher than the theoretical quotients for complete combustion of pyruvate and acetate. Although such over-all data are difficult to interpret because they represent the resultant of many unknown reactions, the high quotient due to pyruvate oxidation is consistent with the synthesis of cellular lipids.

Possible intermediates of fatty acid breakdown

The essentially aerobic character of the breakdown of fatty acids by *Mycobact. ranae* suggested that a process of β-oxidation might operate. Although ketone bodies could not be detected (Rothera test) during the metabolism of butyrate and pentane-1-carboxylate, it was found that *Mycobact. ranae* was able to oxidize acetoacetate, crotonate and β-hydroxybutyrate at considerable speed, the oxidations being strongly inhibited by arsenite (Table 11). The rate of oxidation of acetoacetate is consistent with that of an intermediate in fatty acid catabolism.

Table 11. *Oxidation of acetoacetate, β-hydroxybutyrate and crotonate and the inhibitory effect of arsenite by* Mycobacterium ranae

(Washed bacterial suspension in 3·0 ml. of 0·033 M-phosphate buffer pH 7·4. 38°. Gas: air. Time: 2 hr. Dry wt. of bacteria/cup 7·6 mg. Arsenite 0·0025 M. Inhibition is calculated on the basis of 'extra' oxygen uptake.)

Substrate	Oxygen uptake (μl.) Arsenite absent	Arsenite present	Inhibition (%)
Nil	190	135	—
Acetoacetate (0·01 M)	837	226	94
DL-β-Hydroxybutyrate (0·02 M)	550	157	86
Crotonate (0·02 M)	503	169	89

The oxidation of pentane-1-carboxylate is not completely abolished by iodoacetate or arsenite (Table 5). On this account accumulation of small quantities of acetoacetate is possible in the presence of inhibitors if β-oxidation occurs. Acetoacetic acid, however, could not be found (Rothera test) after restricted oxidation of pentane-1-carboxylate had proceeded for several hours in the presence of iodoacetate or arsenite.

Cell extracts and broken cells

Acetone powders, clear extracts of crushed cells and suspensions of cell debris fail to oxidize pyruvate and fatty acids, although viable cells of *Mycobact. ranae* do so readily. Attempts to secure active preparations by crushing in the presence of cysteine were nugatory. Failure also attended efforts to reconstitute the systems responsible for the oxidation of pyruvate and acetate by addition of catalytic agents such as cozymase, cocarboxylase, adenosinetriphosphate, fumarate, cytochrome *c* (heart muscle) and divalent ions (Mg^{++}, Mn^{++}, Co^{++}). Autoxidizable hydrogen carriers such as methylene blue, pyocyanin and riboflavin did not activate the preparations.

In so far as it is labile and cyanide-sensitive, the enzyme system which oxidizes pyruvate and acetate in *Mycobact. ranae* resembles the glucose-oxidizing system of *Mycobact. phlei* (Edson & Hunter, 1947). Both organisms contain a cytochrome system akin to that of baker's yeast (Todd, 1949).

DISCUSSION

An investigation of the metabolism of *Mycobact. ranae* has made it possible to test certain hypotheses, which have developed from a survey of respiration in acid-fast species (Edson & Hunter, 1943; Franke & Schillinger, 1944), from a more detailed study of *Mycobact. phlei* (Edson & Hunter, 1947) and from additional unpublished work on the metabolism of *Mycobact. phlei* and *Mycobact. smegmatis*.

So far as the observations go, the metabolic pattern of the genus *Mycobacterium* is strictly aerobic; the various species oxidize the same substrates, and their activities differ in degree rather than in kind. The metabolism leads to formation of major cellular components, including lipids which are peculiar to the genus. One feature of the metabolism which is re-emphasized by this study of *Mycobact. ranae* is partial oxidation of substrates by washed suspensions (Table 3). In this respect the Mycobacteria conform to the general conception of Clifton's (1946) assimilation hypothesis, oxidative catabolic reactions being coupled to synthetic reactions. The primary task in elucidating the meta-

bolism of the acid-fast bacteria is to discover means of dissociating the catabolic reactions from the complex without destroying or inhibiting too many enzymic activities.

Iodoacetate has proved to be an agent of the desired type, since small concentrations act selectively on *Mycobact. ranae*, inhibiting the oxidation of acetate almost completely without abolishing the catabolism of pyruvate. Under these circumstances washed suspensions convert pyruvate into acetate (and CO_2) in a manner which is almost quantitative (Table 9). Unpoisoned cells, however, rapidly oxidize pyruvate and acetate at approximately equal rates. For these reasons it may be assumed that acetate is an intermediary in the normal breakdown of pyruvate, although there is as yet insufficient evidence to show how much of the pyruvate takes this pathway. An hypothesis consistent with the facts presented in this paper is illustrated in Fig. 2.

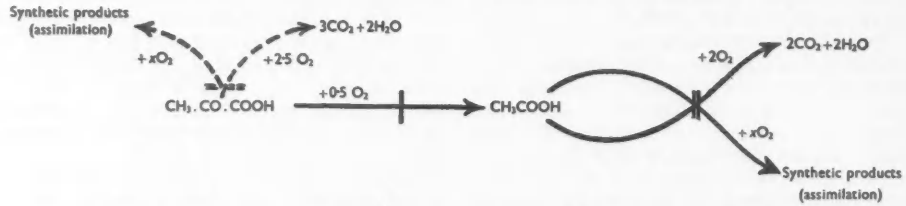

Fig. 2. Scheme of pyruvate and acetate metabolism in Mycobacteria. Reaction blocked by arsenite I, by iodoacetate, arsenite and hydroxylamine II, by arsenite and iodoacetate II.

Several additional observations are relevant to the scheme: (1) the molecular ratio of oxygen uptake/acetate or pyruvate disappearance is less than that required for total combustion (Table 3); (2) the metabolism of added pyruvate or acetate is abolished by arsenite (Tables 5 and 6) as it is in *Mycobact. phlei* (Edson & Hunter, 1947); (3) extra oxygen uptake due to the presence of pyruvate is not affected significantly by hydroxylamine, whereas that due to acetate is inhibited almost completely (Tables 5 and 6); and (4) the utilization of pyruvate is much greater in the absence of iodoacetate than in its presence (Table 9) when the sole reaction occurring is

$$CH_3COCOOH + \tfrac{1}{2}O_2 \rightarrow CH_3COOH + CO_2.$$

It follows that the above reaction is sensitive to arsenite.

The observations can be explained if iodoacetate interferes not only with the enzymes responsible for combustion of acetate but also with coupled reactions of assimilation. When acetate is not oxidized, it is not metabolized in any other way: thus acetate assimilation is blocked under anaerobic conditions and by arsenite, iodoacetate and hydroxylamine. When a portion of added pyruvate is oxidized to acetate in the presence of iodoacetate, assimilation of the excess pyruvate fails (Table 9). The simplest hypothesis which will embrace these facts is indicated by the continuous arrows in Fig. 2, where pyruvate is shown to break down to acetate, the assimilation of which is linked to its own combustion. The experiments, however, do not exclude the possibility of pyruvate being assimilated as a 3-carbon compound in reactions linked to the oxidation of acetate; nor do they exclude an independent oxidative assimilation of pyruvate, linked to its own combustion and paralysed by arsenite and iodoacetate but not by hydroxylamine. Although hydroxylamine blocks the metabolic route via acetate, it does not hinder oxygen consumption in the presence of pyruvate (Table 6). For this reason an alternative assimilation of pyruvate has been shown by dotted arrows in Fig. 2.

Up to the present, efforts to pursue the analysis by means of broken cells or cell extracts have been unsuccessful, due, perhaps, to lability of the enzymes including the cytochrome system. It is evident that elucidation of the coupled reactions, which are probably the key to an understanding of the metabolism of acid-fast bacteria, must await the preparation of pyruvate oxidases as amenable to experiment as those obtained from *Neisseria gonorrhoeae* (Barron & Hastings, 1933), *Lactobacillus delbrückii* (Lipmann, 1939), *Escherichia coli* (Still, 1941) or *Proteus vulgaris* (Stumpf, 1945).

Oxidation of higher homologues of acetic acid is rapid and accompanied by assimilation (Table 3). The enzymes are labile and the mechanism of fatty acid breakdown is obscure since no intermediates have been detected, although acetoacetic acid is oxidized at a rate compatible with the function of an intermediary metabolite (Table 11). The endogenous formation of acetic acid under the influence of iodoacetate is also noteworthy, but speculation regarding the precursor would be premature.

SUMMARY

1. *Mycobacterium ranae* oxidizes pyruvate and acetate at approximately equal rates, which are about four times greater than the rate of endogenous

respiration. Other *n*-fatty acids (up to C_6) are oxidized at even faster rates.

2. The action of inhibitors on the metabolism of *Mycobact. ranae* has been investigated, notably that of iodoacetate which suppresses metabolism of acetate without completely inhibiting the dissimilation of pyruvate.

3. In the presence of iodoacetate, pyruvate is oxidized to acetate quantitatively, assimilation processes being abolished. These facts have been used to construct a hypothesis of normal pyruvate metabolism.

4. Preparations of broken cells and cell extracts were unable to oxidize pyruvate or acetate.

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